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Beta-Endorphin Receptors on Equine Lymphocytes: Relationship to Immune Function and Exercise.

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BETA-ENDORPHIN RECEPTORS ON EQUINE LYMPHOCYTES: RELATIONSHIP TO IMMUNE FUNCTION AND EXERCISE

A DISSERTATION

Submitted to the Graduate Faculty of the
Louisiana State University and Agricultural
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in

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by

Jena Gosselink Hamra
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ABSTRACT

Current evidence suggests that endogenous opioids are released in response to various stressors and that these opioids interact with cells of the immune system, and modulate immune function. However, data substantiating this are conflicting. Therefore, this study endeavored to clarify the role of beta-endorphin (BE) in exercise and immune function.

Beta-endorphin exhibited a diurnal rhythm in the horse with peak plasma levels occurring at 0900 hours. Analgesia, tachycardia and mydriasis were also observed at this time, mimicking the effects of morphine in the horse. Therefore, subsequent experiments were performed at 0900 hours during times of increased basal opioid activity.

Beta-endorphin levels were increased in response to maximal exercise in the horse. The BE response to exercise was attenuated by physical conditioning and was correlated with exercise intensity. Analgesia was noted in unfit horses but not in fit horses after a standardized exercise bout, suggesting that unfit horses experience greater stress when exercised. Naloxone administration resulted in prolonged increases in BE levels following exercise in fit and unfit horses. Naloxone administration also reversed the analgesia noted in fit horses following a more intense exercise bout, but caused hyperalgesia in unfit horses. These results suggest that BE release is controlled through autoreceptors at the hypothalamic or pituitary level and that chronic exercise causes a downregulation of opioid receptors.

Specific, saturable receptors for beta-endorphin were identified on equine

lymphocytes which appeared to be a mu/delta receptor complex, with a K_d of 17 pM and B_{max} of 0.538 fmoles/10^6 cells. Acute exercise resulted in an increased affinity and decreased number of receptors, while chronic exercise resulted in a decreased affinity of the receptor for the ligand. Beta-endorphin suppressed the proliferative response of equine lymphocytes to mitogens through interaction with opioid receptors. Naloxone also suppressed the proliferative response, presumably through opioid receptors.

In conclusion, BE modulates equine immune function through interaction with opioid receptors on equine lymphocytes. Beta-endorphin is also a possible mediator of the effects of acute and chronic exercise on equine immune function, as demonstrated by up- and downregulation of opioid receptors on equine lymphocytes.

CHAPTER ONE

INTRODUCTION

Endogenous opioids are a group of peptide neurohormones or neurotransmitters distributed throughout the central nervous system, pituitary gland and other peripheral tissues including the adrenals. The actions of endogenous opioids, such as beta-endorphin, have been associated primarily with the central nervous system and pain modulation. However, endogenous opioids may also be involved in reproduction, cardiovascular control, endotoxic shock, thermoregulation and, as shown most recently, regulation of immune function. The physiologic effects of the endogenous opioids are mediated through interactions with highly specific membrane receptors. These receptors are thought to be the same receptors that mediate the effects of morphine and other opiate substances [Akil, *et al.*, 1984].

Stress has been defined as "the result produced when an organism is acted upon by physical or psychological forces that disrupt equilibrium or produce strain" [Thomas, 1985]. This term is commonly used to describe biological responses to novel or threatening situations. Stress is capable of influencing immune function and tumor growth. Usually, stress has been associated with depression of the immune system, although, depending on the timing and duration of the stressful stimuli, enhancement of immune function may be seen [Monjan and Collector, 1977; Riley, 1981]. The endogenous opioids are now thought to be one of the potential mediators of stress-induced immunomodulation. Early studies defined the role of the hypothalamic-pituitary-adrenal

axis, particularly the corticosteroids, in immune function in response to stress. Recent studies have demonstrated that beta-endorphin is co-released from the pituitary with adrenocorticotropin (ACTH) in response to various forms of stress, such as exercise and surgery [Cohen, *et al.*, 1986; Guillemin, *et al.*, 1977].

The role of stress in immune function appears to require an interaction between the brain and the immune system. Current research suggests that bidirectional communication exists between the immune and the central nervous systems [Morley, *et al.*, 1987]. Receptors for opioid peptides have been identified on human and murine leukocytes. A number of *in vitro* studies have demonstrated the ability of opioids to influence immune function, such as lymphocyte proliferative responses, natural killer cell activity and granulocyte activity. Recent research has also demonstrated the production of beta-endorphin and ACTH by cells of the immune system, along with the expression of the endorphin and enkephalin opioid genes [Sibinga and Goldstein, 1988].

Although it appears that endogenous opioids are released in response to various forms of stress and in turn interact with cells of the immune system thereby modulating immune function, data substantiating this are conflicting. Opioid binding studies are inconclusive, with evidence of both opioid and non-opioid receptors on immune cells. Few investigators have reported binding constants, receptor numbers or competitive receptor binding data. The ability of opioid peptides to influence leukocyte function varies with the opioid peptide, leukocyte cell type, and species. Here again, evidence of both opioid and non-opioid effects on leukocyte function have been reported [Sibinga and Goldstein, 1988].

Exercise, as a form of exertional stress, has commonly been used as a method of testing physiological responses and adaptations. It can be easily quantified and repeated. Exercise has been used in both humans and animals to evaluate the release of endogenous opioids. A number of studies in humans have demonstrated increased circulating beta-endorphin levels following maximal exercise bouts [Farrell and Gustafson, 1986]. Li and Chen [1987] have also demonstrated this phenomenon in thoroughbred horses.

Exercise is particularly stressful in an unfit or unconditioned animal. Through athletic conditioning, the stressfulness of intense exercise can be modified in both human and equine athletes. Stress, and its effect on immune function, are important in the overall physical condition of any animal. Therefore, exertional stress, such as exercise, may modulate immune function and influence susceptibility to disease.

The purpose of this study was to clarify the role of peripherally circulating beta-endorphin in immune function as it relates to exercise. Thoroughbred horses can be used as a model of human athletes because they exercise and compete in a similar manner. The role of beta-endorphin in immune function and exercise was assessed by evaluating beta-endorphin levels in response to both acute and chronic exercise, characterizing beta-endorphin receptors on equine lymphocytes, and evaluating lymphocyte proliferative responses in the presence of beta-endorphin. Beta-endorphin levels and beta-endorphin receptor binding were evaluated in populations of sedentary, conditioned (or fit) horses, and in fit horses following exercise. By measuring these parameters, the effects of both acute and chronic fluctuations in circulating beta-endorphin levels on equine lymphocyte receptor binding were evaluated. Collectively, these studies contributed to the elucidation

of the role that beta-endorphin plays in modulating immune function at the cellular level and how exercise and conditioning alter the ability of beta-endorphin to modulate immune function.

The overall objective of this project was to clarify the role of peripherally circulating beta-endorphin in immune function as it relates to exercise in the horse. The specific objectives of this project were:

1. Determination of optimal sampling times:

Diurnal variation in plasma beta-endorphin levels and nociceptive thresholds have been reported in rats and mice. Plasma beta-endorphin levels also demonstrate a diurnal rhythm in humans with peak levels occurring at 0800 hours [Petraglia, *et al.*, 1983]. This diurnal rhythm influences the quantitative response to stress, suggesting that the opioid system has tonic activity, that is, greater response when the system is already active [Wesche and Frederickson, 1979]. Therefore, plasma beta-endorphin levels and nociceptive thresholds were measured over 24 hours to establish a diurnal rhythm and optimum experimental sampling times for this study.

2. Determination of beta-endorphin levels after maximal exercise in sedentary and fit horses:

To determine how maximal exercise influences circulating beta-endorphin levels in sedentary and fit horses, beta-endorphin levels were assessed after a maximal exercise session in sedentary horses and again after a nine week period of physical conditioning

or training. Naloxone pretreatment before exercise was used to evaluate opioid receptor involvement in the animal's response to maximal exercise. Pain threshold measurements, heart rate and lactate levels were used to assess the degree of exertional stress produced by each exercise session. Fitness and exercise intensity were determined by heart rate, respiratory rate and lactate levels.

3. Development of a radioligand binding assay for beta-endorphin receptors on equine lymphocytes:

A radioligand binding assay was developed which allowed for identification and characterization of beta-endorphin receptors on equine lymphocytes.

4. Determination of the binding parameters of beta-endorphin receptors in a population of normal sedentary horses and in fit horses before and after a maximal exercise session:

Binding parameters in normal sedentary horses were determined using the radioligand binding assay previously developed. The same assay was used to assess the binding parameters in fit horses prior to and immediately following a maximal exercise session. The binding parameters of the fit horses at rest were then compared to those of the sedentary horses. These measurements provided a means for assessing the effects of acute and chronic exercise on equine lymphocyte beta-endorphin receptor regulation.

5. Assessment of beta-endorphin modulation of lymphocyte function:

Beta-endorphin modulation of lymphocyte function was determined by measuring

the proliferative response of equine lymphocytes to various mitogens in the presence of beta-endorphin. Here again, the effects of naloxone were used to evaluate the involvement of opioid receptors.

6. Correlation of the binding study results with beta-endorphin levels and lymphocyte function analysis:

The results of the binding studies were correlated with beta-endorphin levels in sedentary and fit horses before and after maximal exercise. These results were then analyzed in relation to the lymphocyte blastogenesis assay results in order to determine the role beta-endorphin receptors play in exercise, physical conditioning and immune function.

CHAPTER TWO

LITERATURE REVIEW

Beta-endorphin is an endogenous opioid peptide which is commonly associated with the central nervous system and pain modulation. Since this peptide is not thought to cross the blood brain barrier, its function in the peripheral circulation is a matter of speculation [Banks and Kastin, 1987]. Beta-endorphin is co-released along with ACTH into the bloodstream in response to a variety of stressful stimuli [Guillemin, *et al.* 1977]. Stress is capable of altering immune function. Exercise, as a form of exertional stress, has also been demonstrated to influence immune function [Keast, *et al.*, 1988]. Beta-endorphin has been shown to bind to cells of the immune system and influence the function of these cells. It is thought, therefore, that beta-endorphin may be a potential modulator of stress-induced immunomodulation [Sibinga and Goldstein, 1988]. This review will examine current research dealing with beta-endorphin and its role in immune function and exercise.

A. PHYSIOLOGY OF BETA-ENDORPHIN

1. Distribution, Release and Catabolism

Beta-endorphin, a peptide containing thirty-one amino acids, is derived from the precursor molecule proopiomelanocortin (POMC) along with adrenocorticotropin (ACTH), beta-lipotropin and alpha-melanocyte stimulating hormone (alpha-MSH) (Figure 1). Beta-endorphin is the sole product of the precursor proopiomelanocortin that possesses activity

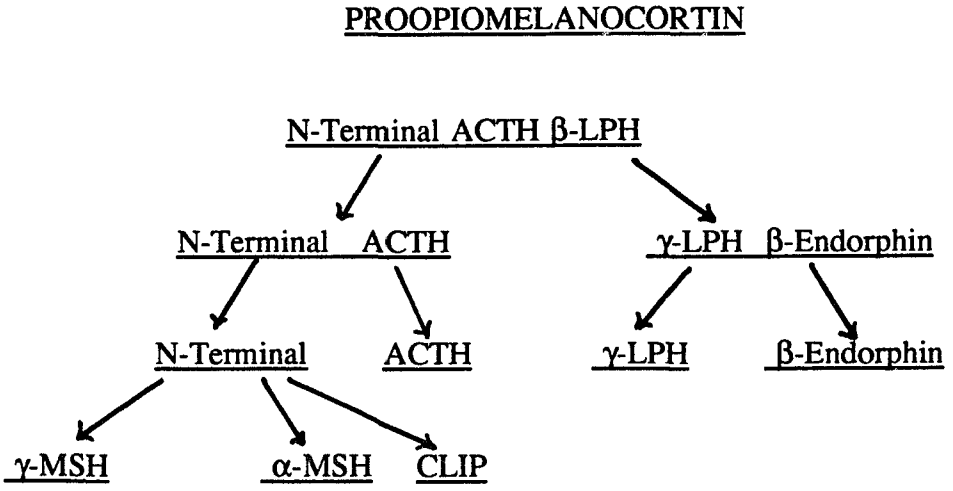

Figure 1. Generation of beta-endorphin from the precursor molecule proopiomelanocortin (POMC). β-lipotropin = β-LPH, γ-lipotropin = γ-LPH, adrenocorticotropin = ACTH, α-melanocyte-stimulating hormone = α-MSH, γ-melanocyte-stimulating hormone = γ-MSH, corticotropin-like intermediate peptide = CLIP. (Adapted from Frederickson and Geary, 1982).

at the opioid receptor. The major site of production of beta-endorphin is in the intermediate and anterior lobes of the pituitary, with greater amounts being produced by the intermediate lobe in most species [Frederickson and Geary, 1982]. Beta-endorphin appears to have a more restricted distribution in the intermediate lobe of the horse than in rats and dogs. Equine beta-endorphin positive cells are located in the intermediate lobe adjacent to the lobus nervosus and are fewer in number than noted in other species, suggesting that one of the functions of beta-endorphin in the horse is regulation of oxytocin release [Amann, *et al.*, 1987]. In the mammalian central nervous system, beta-endorphin is found in the cell bodies and neurons of the arcuate nucleus of the hypothalamus. Fibers from the hypothalamus containing beta-endorphin innervate the median eminence and the dorsal midline thalamus. Additional fibers containing beta-endorphin also innervate the amygdala, preoptic area, ventromedial nuclei, paraventricular nucleus, periaqueductal gray, reticular formation and stria terminalis [Frederickson and Geary, 1982].

Proopiomelanocortin cells in the anterior and intermediate lobes of the pituitary appear to process the precursor POMC differently. In the anterior lobe, the processing results primarily in beta-lipotropin (70%) with unmodified beta-endorphin comprising about thirty percent of the material released. The intermediate lobe stores little or no beta-lipotropin and therefore primarily produces beta-endorphin (97%). The form of beta-endorphin released from the intermediate lobe is not beta-endorphin(1-31) but N-acetyl-beta-endorphin(1-27), a modified form which has no activity at the opioid receptor [Frederickson and Geary, 1982; Akil, *et al.*, 1984].

Circulating levels of beta-endorphin result from release of beta-endorphin by the pituitary, while cerebrospinal (CSF) levels derive from the periventricular system in the brain. A number of factors can cause changes in beta-endorphin levels in plasma and CSF, such as exercise, surgery and stimulation of the periaqueductal gray (Table 1) [Frederickson, 1984]. The release of beta-endorphin from the anterior lobe of the pituitary is coupled with that of ACTH. These two peptides are released from the anterior pituitary in response to the hypothalamic peptide, corticotropin-releasing factor (CRF) [Rivier, *et al.*, 1982]. Although it has been reported that the anterior lobe primarily produces beta-lipotropin, Young, *et al.* [1986] demonstrated preferential release of beta-endorphin from the anterior lobe when stimulated by CRF. These investigators reported that equimolar amounts of beta-endorphin and beta-lipotropin were released from an unstimulated anterior lobe, while stimulation by CRF produced two-fold more beta-endorphin than beta-lipotropin. The CRF neurons also appear to interact with gamma-aminobutyric acid (GABA) neurons since GABA receptor agonists exert inhibitory effects on beta-endorphin and beta-lipotropin release from the anterior lobe of the pituitary [Petraglia, *et al.*, 1986]. Adrenal steroids regulate the release of ACTH and beta-endorphin through negative feedback by inhibiting stimulated but not basal release [Frederickson and Geary, 1982]. Beta-endorphin release from the intermediate lobe of the pituitary appears to be regulated differently from that of the anterior lobe. Dopamine and dopaminergic agents inhibit release, but CRF, adrenal steroids and GABA-ergic mechanisms have little or no effect [Frederickson and Geary, 1982; Petraglia, *et al.*, 1986].

TABLE 1. Factors That Influence Beta-endorphin Levels in CSF or Plasma

	Increase	Decrease
Human CSF	Stimulation of PAG for pain relief Electroacupuncture for pain relief Heroin addiction	Cushing's disease
Human plasma	Surgical stress Exercise stress Heroin addiction Labor/delivery Adisson's disease Cushing's disease Morphine	Dexamethasone
Rodent plasma	Noxious stress Morphine Naloxone Vasopressin Adrenalectomy Insulin	Chronic morphine Hypophysectomy Dexamethasone

*Adapted from Frederickson [1984].

Stress appears to alter the pattern of release of both beta-endorphin and ACTH. Acute stress results in decreased responsiveness to CRF, while chronically stressed rats demonstrate increased ability to release ACTH and beta-endorphin in response to CRF [Young and Akil, 1985a]. In fact, chronically stressed rats show increased amounts of ACTH and beta-endorphin stored in the anterior pituitary [Young and Akil, 1985b]. Stress also appears to cause preferential release of beta-endorphin over beta-lipotropin from the anterior pituitary [Young, *et al.*, 1986]. Pretranslational and posttranslational mechanisms for regulating release of beta-endorphin and ACTH are altered by stress. Shiomi, *et al.* [1986] have demonstrated acceleration of the rate of translation of POMC after acute stress and an increase in the transcription of POMC messenger RNA after chronic stress. Hollt, *et al.* [1986] also noted increased levels of POMC messenger RNA after chronic intermittent foot-shock in rats. Stress also modifies the release of beta-endorphin from the intermediate lobe of the pituitary. Chronic stress induces increased synthesis and release of the opioid-inactive N-acetylated beta-endorphin(1-31) [Akil, *et al.*, 1985].

The biological activity of beta-endorphin is dependent upon its structure. Acetylation or removal of the N-terminal tyrosine results in loss of activity at opioid receptors. If beta-endorphin is shortened at the C-terminal, as with alpha-endorphin or gamma-endorphin, there is also loss of activity. An enzyme has been identified which is specific for the leu^{77}-phe^{78} site. The action of this enzyme results in the formation of gamma-endorphin from beta-endorphin. Another aminopeptidase has been purified from rat brain and has been shown to release tyrosine from alpha, gamma and beta-endorphin

[Frederickson and Geary, 1982]. The regulation of the catabolism of beta-endorphin is poorly understood and requires further investigation.

2. Structure and Receptor Function

Beta-endorphin is a thirty-one amino acid peptide which is derived from the precursor molecule proopiomelanocortin (POMC). This peptide differs slightly between species with the main differences occurring at the carboxy terminus. Equine beta-endorphin differs from human by three amino acids (Figure 2) [Li, *et al.*, 1981]. The sequence of amino acids is important in determining the receptor specificity of the particular opioid peptide. All opioid peptides share the same amino acid sequence of Tyr-Gly-Gly-Phe-Met(or Leu) at the N-terminus [Akil, *et al.*, 1984]. Binding to opioid receptors is traditionally thought to occur through the N-terminal region of the molecule. The C-terminal sequence is thought to convey potency and provide stability against exopeptidase attack while the N-terminal sequence is necessary for opioid activity [Deakin, *et al.*, 1980; Evans, *et al.*, 1986].

The sites of action for the opioid peptides are the "opiate" receptors. The existence of multiple forms of opioid receptors was first proposed by Martin, *et al.* [1976]. The basic types are designated as the mu or morphine receptor, the kappa or ketocyclazocine receptor, the sigma or SKF-10047 receptor and the delta or enkephalin receptor [Akil, *et al.*, 1984]. Thus, the enkephalins, whose structure contains the first four amino acids in beta-endorphin, prefer the delta receptor while beta-endorphin binds equally well at the mu or delta receptor (Table 2) [Sforzo, 1989]. It has been postulated

```
                1                   5                  10
      Human:  H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-
Camel, Bovine: H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-
      Equine:  H-Tyr-Gly-Gly-Phe-Met-Ser-Ser-Glu-Lys-Ser-

                          15                  20
          Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-
          Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-
          Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-

                      25                      31
          Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu-OH
          Ala-Ile-Ile-Lys-Asn-Ala-His-Lys-Lys-Gly-Gln-OH
          Ala-Ile-Ile-Lys-Asn-Ala-His-Lys-Lys-Gly-Gln-OH
```

Figure 2: Comparative amino acid sequences of human, camel, bovine, and equine beta-endorphin. (Adapted from Li, et al., 1981)

TABLE 2. Opioid Ligand Interactions With Opioid Receptor Subtypes

Ligand	Delta	Mu	Kappa
Agonists			
Met-enkephalin	High	Low	Low
Beta-endorphin	High	High	Low
Dynorphin	Low	Low	High
Morphine	Low	High	Low
Antagonists			
Naloxone	Low	High	Low

*Adapted from Sforzo [1989].

that the N-terminal tetrapeptide sequence, Tyr-Gly-Gly-Phe, is the sequence responsible for triggering the receptor response while the C-terminal sequence is responsible for receptor subtype specificity in addition to possibly conveying potency [Schwyzer, 1986]. The different forms of opioid receptors are each associated with specific physiologic actions. The physiologic actions associated with mu, kappa and sigma receptors were demonstrated by Iwamoto and Martin [1981] in the chronic spinal dog. Later, with the discovery of the enkephalins, a distinct receptor, the delta receptor, was proposed. Further investigation revealed that specific physiological actions of morphine could be attributed to subclasses of the mu receptor or the delta receptor (Table 3) [Goodman and Pasternak, 1984].

Gero [1986] has proposed that opioid receptors are flexible and that agonists distort the receptor thus activating it. Drugs such as antagonists attach without distortion, and therefore have no effect. Other studies suggest that the opioid receptors are in fact one receptor which is "interconvertible" or that they exist in close proximity on the cell surface membrane in a manner which allows interaction [Morley, 1983; Lee and Smith, 1980]. It has also been proposed that the opioid receptor is composed of both protein and lipid. The protein contains the binding site for the enkephalins while the lipid contains the site for the alkaloids, such as morphine. Beta-endorphin is proposed to interact with both sites [Lee and Smith, 1980]. Bowen, *et al.* [1981] have postulated that the mu and delta forms of the opioid receptor "interconvert" when the mu receptor couples to adenylate cyclase, while Schwyzer [1986] has suggested that the opioid peptide receptor sites are regulated by peptide membrane interactions. He proposed that the delta site is

TABLE 3. Actions At Opiate Receptors

Mu_1	Mu_2	Delta	Kappa	Sigma
Supraspinal analgesia	Bradycardia	Analgesia	Miosis	Mydriasis
Prolactin release	Respiratory depression	Respiratory depression	Analgesia	Tachy-cardia
Catalepsy	Decreased gastro-motility	Modulation of dopamine turnover		
Hypothermia		Reversal of endotoxic shock		
Miosis				

*Adapted from Goodman and Pasternak [1984].

exposed to the aqueous compartment surrounding the target cell and is in a cationic vicinity while the mu site is exposed to the anionic fixed-charge aqueous compartment of the membrane and that the kappa site is buried in the hydrophobic membrane compartment. Recently, evidence for a mu/delta opioid receptor complex with interacting mu and delta binding sites in rat striatal membranes has been demonstrated [Schoffelmeer, *et al.*, 1990].

Using antibodies directed against the opioid receptor, several investigators have begun to identify opioid receptors by isolating subunits of the receptor. Maneckjee, *et al.* [1988] have identified a subunit of the opioid receptor with a M_r of 94,000 daltons which they propose to be an opioid recognition unit. Analysis of opioid receptors on neuroblastoma X glioma cells has shown that the receptor is a complex with a M_r of 210,000 daltons containing four polypeptide chains with M_r of 68,000, 58,000, 45,000 and 30,000 daltons. A leukocyte opioid receptor which is virtually identical to that on neuroendocrine cells has also been demonstrated [Carr and Blalock, 1989]. Affinity cross-linking experiments in bovine brain membranes result in labeling of a 65,000 dalton and a 53,000 dalton protein in which labeling was reduced by a mu agonist and a delta agonist, respectively, suggesting that the mu and delta sites are separate proteins possessing different molecular weights. In contrast, affinity cross-linking of the mu/delta complex in rat striatal membranes exhibited labeling of a single 80,000 dalton glycoprotein in which labeling was only partially (50%) reduced by selective mu and delta agonists [Schoffelmeer, *et al.*, 1990]. It therefore appears that the mu and delta binding sites may exist independently or in a physically associated manner. Whether or

not they derive from the same gene and result from different posttranslational modifications has yet to be determined.

Finally, endogenous opioids appear to modulate cell activity through inhibition of adenylate cyclase activity thereby inhibiting cyclic AMP accumulation [Klee and Nirenberg, 1976]. The endogenous opioids appear to convert adenylate cyclase to a form with altered activity and thereby alter the relative amounts of low and high activity forms of the enzyme [Sharma, *et al.*, 1977]. Zukin and Tempel [1986] have suggested that upregulation of the opioid receptor is accompanied by an increased coupling to the inhibitory guanyl nucleotide binding protein and that downregulation of the receptor involves dissociation from the nucleotide binding protein. Therefore, opioid peptides modulate signal transduction mechanisms by altering adenylate cyclase and guanyl nucleotide binding protein activity, thus controlling second messenger systems. This research appears to indicate a generally inhibitory role for the endogenous opioids.

3. Diurnal Variation

Plasma levels of beta-endorphin demonstrate a diurnal rhythm which coincides with that of plasma ACTH, beta-lipotropin, and cortisol, with the highest levels occurring at 0800 hours in normal humans [Petraglia, *et al.*, 1983; Gil-Ad, *et al.*, 1986; Farsang, *et al.*, 1983]. Beta-endorphin levels in human and monkey cerebrospinal fluid (CSF) also vary diurnally with the highest levels occurring between 0600 and 0800 hours [Barreca, *et al.*, 1986; Naber, *et al.*, 1981]. A diurnal rhythm of proopiomelanocortin (POMC) messenger RNA has been demonstrated in the intermediate pituitary lobe of rats. The

variation of POMC messenger RNA parallels that of beta-endorphin and these events are preceded by similar changes in the POMC gene transcription [Millington, *et al.*, 1986]. Wesche and Frederickson [1979] suggested that the endogenous opioid system exhibits tonic activity. They demonstrated higher levels of endogenous opioid peptides in mice after a noxious stimulus in the afternoon as opposed to the morning. Here again, increased activity was observed at a time when the endogenous opioid system was the most active. These findings are in conflict with those of Kant, *et al.* [1986], who were unable to demonstrate increased release of beta-endorphin in response to stress during the dark phase in rats.

A diurnal rhythm in endogenous opioids suggests that they may play a role in the diurnal fluctuation of nociceptive responses. Rats and mice both demonstrate decreased sensitivity to pain at night [Wright, 1981; Frederickson, *et al.*, 1977]. This coincides with the diurnal rhythm of endogenous opioids since both the rat and mouse are nocturnal animals with higher opioid levels occurring at night [Tang, *et al.*, 1984]. Henry [1981] demonstrated increased activity of dorsal horn units in the spinal cat following naloxone administration during the day and early evening. Administration of naloxone at night did not produce the excitation seen during the day in cats which were conditioned to a regular day/night routine. This investigator suggested that the increase in activity observed was due to competition with endogenous opioids by naloxone, proposing therefore that higher opioid levels in the day led to an increased observable effect. Evaluation of nociceptive thresholds in man has produced conflicting results. The time of day at which the pain threshold was the highest varied from 0300 hours to 1800 hours [Morawetz, *et al.*, 1984].

Morawetz, *et al.* [1984] suggested that the technique used to measure pain thresholds may influence the results leading to erroneous assumptions of a diurnal rhythm. Only one human study attempted to correlate beta-endorphin levels with variations in pain threshold and they were unable to demonstrate a significant correlation [Sandrini, *et al.*, 1986].

In summary, beta-endorphin is a thirty-one amino acid peptide which is released from the anterior and intermediate lobes of the pituitary in response to stress. Stress alters the pattern of releasability by causing preferential release of beta-endorphin from the anterior lobe of the pituitary. Chronic stress also causes increased storage of beta-endorphin in the pituitary of rats. Beta-endorphin binds equally well to the mu and delta opioid receptors and appears to exert its action through inhibition of adenylate cyclase activity. Identification of opioid receptors has demonstrated very similar characteristics between opioid receptors in both the central nervous system and the immune system. Beta-endorphin levels exhibit a diurnal rhythm both in plasma and cerebrospinal fluid. Although the evidence is conflicting, the diurnal variation of beta-endorphin may follow that of pain threshold. Beta-endorphin release seems to exhibit tonic rather than phasic activity and therefore increased release of beta-endorphin in response to stress or other stimuli may occur at times when basal activity is highest.

B. BETA-ENDORPHIN AND EXERCISE

1. Exercise-induced Changes in Beta-endorphin Levels

Increasing interest in the role of endogenous opioids in exercise and training has

led to an explosion of research into this area. Early studies demonstrated increased plasma beta-endorphin levels following "strenuous" exercise in humans [Colt, *et al.*, 1981]. These studies did not quantify exercise intensity and therefore were unable to demonstrate any relationship between exercise intensity and the levels of beta-endorphin released. Most investigators used radioimmunoassay procedures to measure beta-endorphin levels. Unfortunately, beta-endorphin exhibits close structural similarity to beta-lipotropin and therefore could not always be differentiated from beta-lipotropin by available radioimmunoassay procedures. Thus, many investigators were and are measuring beta-endorphin-like immunoreactivity rather than actual beta-endorphin levels. The different methods used to measure beta-endorphin levels have led to reports of beta-endorphin levels varying from 10 pg/ml to 1.6 ng/ml [Farrell, 1985]. Therefore, while many of these studies may correctly indicate a change in beta-endorphin levels, the exact magnitude of change may not be accurate [Sforzo, 1989].

Current studies suggest that beta-endorphin levels increase significantly after moderately high intensity exercise (i.e. 80% VO_{2max}) or maximal exercise intensity (i.e. graded exercise test to exhaustion) [Gambert, *et al.*, 1981; Goldfarb, *et al.*, 1987]. Submaximal exercise intensity (i.e. 60% VO_{2max} or lower) does not alter beta-endorphin levels significantly [Rahkila, *et al.*, 1987; Langenfeld, *et al.*, 1987]. The exercise stress threshold required to stimulate release of beta-endorphin has been reported to occur between 50 and 75% VO_{2max} or at exercise intensities greater than 80% VO_{2max} [Donevan and Andrew, 1987; Rahkila, *et al.*, 1988]. There also appears to be no difference in beta-endorphin release between trained men and women [Rahkila, *et al.*, 1987]. Therefore, it

appears that high exercise intensity is required to significantly elevate beta-endorphin levels and is able to do so in as little as thirty to sixty seconds with peak levels two to ten fold greater than baseline. Beta-endorphin levels continue to increase for five to fifteen minutes post exercise and return to baseline within an hour of cessation of exercise. There is also considerable interindividual and intraindividual variation in the beta-endorphin response to exercise. The endorphin response to exercise does not appear to be linear but perhaps is best described as a curvilinear response which closely resembles the exercise-induced blood lactate accumulation curve [Sforzo, 1989].

Recent research suggests that exercise training results in augmented beta-endorphin release in response to high intensity exercise. Originally, Carr, *et al.* [1981] demonstrated increased plasma levels of beta-endorphin in response to exercise in trained individuals. This work was supported by Farrell, *et al.* [1987] and Mougin, *et al.* [1987] who both demonstrated greater peak beta-endorphin levels following supramaximal exercise in trained athletes versus untrained subjects. The question arises as to whether the absolute exercise intensity alone, which is attainable only by trained athletes, is responsible for the increased levels of beta-endorphin noted in these experiments. Interestingly, Howlett, *et al.* [1984] showed no change in levels of beta-endorphin released in trained individuals following exercise tests at 80% VO_{2max} as compared to untrained individuals, although Bullen, *et al.* [1984] demonstrated incremental increases in the peak response to an exercise test at 85% VO_{2max} as training progressed. Metzger and Stein [1984] also were unable to demonstrate any change in the releasability of beta-endorphin following exercise in trained rats. However, these investigators did demonstrate lower basal levels of beta-

endorphin in the trained animals.

Despite the fact that numerous studies have been performed in order to elucidate the role of peripheral opioids in exercise, only a few studies have examined central opioid function as it pertains to exercise. Decreased levels of hypothalamic beta-endorphin have been reported following a 20-30 minute swim in cold water in rats [Wardlaw and Frantz, 1980]. However, Metzger and Stein [1984] were unable to detect any difference in brain levels of beta-endorphin after sprint running in rats. Another study performed in rats was unable to demonstrate any change in brain beta-endorphin levels following exercise in trained versus untrained animals, although this study did demonstrate increased levels of beta-endorphin in the nucleus accumbens following exercise to fatigue in all animals [Blake, *et al.*, 1984]. Houghten, *et al.* [1986] examined brain receptor binding after exercise in rats and found no change in the dissociation constant but decreased receptor number. Sforzo, *et al.* [1986] found enhanced binding of an opioid antagonist in five regions of the brain after 2 hours of warm water swimming in rats but Wagner [1986] was unable to reproduce these results in humans. In humans, treadmill exercise resulted in decreased binding of radiolabeled carfentanil, a potent mu ligand. Although these studies report altered brain opioid activity following exercise there are no uniform results or experimental design, therefore making it difficult to draw conclusions about the significance of these findings.

The correlation between beta-endorphin levels in the bloodstream and the CSF provokes much controversy. While some investigators maintain that beta-endorphin does not cross the blood-brain barrier and therefore peripheral levels are not necessarily

correlated with central levels, others suggest that opioid peptides have sufficient permeability to exert central effects [Rossier, *et al.*, 1977; Rapoport, *et al.*, 1980]. One interesting study attempted to correlate peripheral with CSF levels of beta-endorphin in the dog following both high and low intensity exercise. The peripheral release of beta-endorphin was dose-related with respect to both time and intensity while CSF levels of beta-endorphin increased during low but not high intensity exercise [Radosevich, *et al.*, 1989]. Consequently, it appears that the increase in CSF beta-endorphin was not dependent upon exercise intensity or plasma levels of beta-endorphin.

Beta-endorphin levels following exercise in the horse have been examined by few investigators. Evans *et al* [1985] demonstrated increased plasma beta-endorphin levels in the horse following a strenuous gallop with levels returning to baseline within a half hour. Li and Chen [1987] demonstrated incremental increases in beta-endorphin levels following a trot, slow gallop and fast gallop in Thoroughbred horses. Although Li and Chen [1987] reported approximate speed, none of these investigators reported heart rate, respiratory rate, lactate levels or any other parameters used in evaluating exercise intensity. Also, Evans, *et al.* [1985] used a human radioimmunoassay kit to measure equine plasma beta-endorphin levels without reporting any validation of this method. Despite the fact that Li and Chen [1987] developed a radioimmunoassay for equine beta-endorphin, their antibody was made using porcine beta-endorphin as the immunogen, which differs by two amino acids from equine beta-endorphin [Li, *et al.*, 1981]. The antiserum cross-reacted 15% with beta-lipotropin, 100% with camel beta-endorphin and 60% with porcine beta-endorphin. They did not report the cross-reactivity of equine beta-

endorphin with their antiserum although it differs in amino acid sequence from all those tested. Therefore, although increases in beta-endorphin levels probably do occur following exercise in the horse, the intensity of exercise which stimulates a response and the exact magnitude of the response have yet to be addressed.

2. Exercise-Induced Changes in Pain Threshold

Changes in both central and peripheral levels of endogenous opioids following exercise have led many investigators to suggest that exertional stress is also capable of altering pain threshold through modulation of endogenous opioid function. Following a three minute swim, mice exhibited increased pain thresholds, while swim stress also reduced chronic pain in mice as measured by modification of behavioral responses to subcutaneous formalin injection [Christie, *et al.*, 1981, Carmody and Cooper, 1987]. This effect was abolished by administration of naloxone which suggests an opioid mediated mechanism [Carmody and Cooper, 1987]. After an hour of running, rats trained to run spontaneously in running wheels displayed increased pain thresholds which were also reversed by naloxone [Shyu, *et al.*, 1982].

Investigation into the effect of exercise on pain thresholds in humans has led to conflicting results. Droste, *et al.* [1988] were unable to detect any change in electrical pain thresholds after exercise in patients with silent and symptomatic myocardial ischaemia. These results may have been influenced by the ability of these patients to reach a sufficient level of intensity of exercise since exercise was terminated with angina pectoris. Janal, *et al.* [1984] reported that intense exercise (85% VO_{2max}) produced a

hypoalgesic effect that was reversed by naloxone. These investigators also reported elevated ratings of joy, euphoria, cooperation and conscientiousness after exercise. Of these, only the elevations in joy and euphoria were attenuated by naloxone, suggesting a role for beta-endorphin or some other endogenous opioid in what is popularly called the "runner's high." Surbey, *et al.* [1984] also reported that naloxone decreased pain threshold in human athletes following a maximal exercise test although not significantly. Although these reports suggest that endogenous opioids are involved in increased pain threshold following exercise, there is no definitive reproducible data to support this claim.

3. Evaluation of Exercise Intensity

Exercise intensity is commonly assessed by measuring such variables as heart rate, respiratory rate, maximal oxygen consumption (VO_{2max}), and blood lactate levels. Blood lactate levels are used as an index of anaerobic metabolism with high levels indicating lactate accumulation commonly noted after maximal exercise. The anaerobic threshold is defined as the "point where metabolic acidosis and associated changes in gas exchange in the lungs occur during graded exercise" [Astrand and Rodahl, 1986]. It has also been described as the "onset of blood lactate accumulation" and has been standardized in humans to occur when lactate blood levels reach 2.5 to 4.0 mmol/L [Astrand and Rodahl, 1986]. In horses, lactate levels do not exceed 4 mmol/L after submaximal exercise but increase 20 to 25 fold over baseline after maximal exercise. Therefore, it has been concluded that submaximal work is below anaerobic threshold for skeletal muscle metabolism in Thoroughbred racehorses [Judson, *et al.*, 1983]. As in humans, a blood

lactate level of 4 mmol/L is considered to be the approximate level of exercise induced onset of blood lactate accumulation or anaerobic threshold in horses [Persson, 1983]. In exercising horses, the running speed at which lactate accumulation occurs has been measured as occurring at 10.25-11.75 m/sec [Wilson, *et al.*, 1983].

Blood lactate levels have been shown to be exponentially related to both exercise heart rate and work load (expressed as velocity). The measurement of maximal heart rate provides very reproducible results which can be used to assess the degree of fitness of a horse. At a work load where the heart rate reaches 200 beats/min, blood lactate levels reach those corresponding to anaerobic threshold. Therefore a heart rate of 200 beats/min (HR200) is used to define maximal aerobic work power in the horse [Persson, 1983]. The heart rate recovery curve is also considered to be a good objective assessment of the degree of fatigue experienced by a horse during exercise [Rose, 1983].

Respiration in exercising horses differs from that of humans in that the horse's ability to increase cardiac output and oxygen uptake is greater than that of a human athlete, therefore making respiration a possible limiting factor in equine athletic performance. Also, in horses, breathing frequency is a function of stride frequency. Because of this, during a gallop respiratory frequency and stride frequency become synchronized on a one to one basis in the horse. Therefore, a galloping horse is forced to respire very frequently (up to 140 breaths/min) but with a low tidal volume. This is opposed to a human athlete in which locomotion has little affect on thoracic mechanisms so that in human athletes respiration is independent of step frequency. In a horse, respiration increases from resting values of approximately 35 breaths/min to 120-140

breaths/min in a maximally exercising horse [Gillespie and Pascoe, 1983; Hornicke, *et al.*, 1983].

Heart rate, lactate levels and respiratory rate can be used to evaluate exercise tolerance and fitness. In man, trained or fit athletes have lower muscle and blood lactate levels during submaximal exercise than do untrained individuals. The heart rate response to exercise is also lower in trained athletes [Persson, 1983]. In the horse, blood lactate levels in response to submaximal exercise at 8.5 m/sec decreased significantly throughout a training period [Thornton, *et al.*, 1983]. Training also results in decreased heart rate during exercise as well as a shift in the slope of the post exercise recovery curve in the horse [Fregin and Thomas, 1983; Persson, 1983].

Release of beta-endorphin in humans has been associated with the degree of exercise intensity. Early studies did not accurately measure exercise intensity, thereby leading to conflicting and often confusing results. As interest and research in this area increased, the release of beta-endorphin has been linked with an exercise intensity of 75-80% VO_{2max} [Sforzo, 1989]. Several investigators have suggested that there is a connection between the onset of blood lactate accumulation and the release of beta-endorphin in humans. De Meirleir, *et al.* [1986] noted that after one hour of submaximal work below anaerobic threshold (4 mmol/l), beta-endorphin levels did not change. Beta-endorphin levels increased with high intensity exercise, with the rise in lactate levels above anaerobic threshold always preceding the rise in beta-endorphin. Mougin, *et al.* [1987] reported that the increase in beta-endorphin following exercise was significantly correlated with that of plasma lactate levels after a nordic ski race. They also noted that

experienced trained skiers showed greater beta-endorphin and lactic acid levels than did the recreational skiers. Rahkila, *et al.* [1988] also reported that increases in beta-endorphin levels following exercise were always accompanied by corresponding increases in blood lactate levels.

In summary, intense exercise (75-80% VO_{2max}) stimulates the release of beta-endorphin into the peripheral circulation. Due to the different methods used to measure beta-endorphin levels, the exact magnitude of change measured varies greatly between studies. Current research suggests that exercise training results in augmented beta-endorphin release in response to exercise although not all studies support this theory. Despite the fact that most studies do detect changes in central opioid function following exercise, the lack of uniform results or experimental design has led to confusing and conflicting reports. Beta-endorphin levels also increase after exercise in the horse, although the intensity of exercise required to elicit a response and the magnitude of the response needs to be clarified. Intense exercise appears to alter pain perception through opioid mechanisms despite the conflicting reports from human experiments. The release of beta-endorphin appears to be correlated with the onset of blood lactate accumulation and exercise intensity above anaerobic threshold. Exercise intensity and fitness of the athlete may be evaluated by blood lactate levels, heart rate and respiratory rate. Lactate levels above 4 mmol/L indicate anaerobic threshold is surpassed in the horse and combined with a heart rate of approximately 200 beats/min can be used to indicate maximal work effort on the part of the animal. Fitness can be evaluated by decreased

lactate levels, heart rate and respiratory rate and increased recovery to resting values.

C. BETA-ENDORPHIN AND IMMUNE FUNCTION

1. Opioid Receptors on Cells of the Immune System

A theory of interaction between the central nervous system and the immune system has emerged from current research. One finding which supports this theory is the existence of opioid receptors on cells of the immune system, particularly lymphocytes. Lymphocytes can be divided into two main subsets: T cells and B cells. T cells are derived from the thymus and regulate the proliferation and differentiation of B cells into antibody secreting plasma cells through secretion of interleukins and interferons. B cells are derived from the bone marrow and synthesize and secrete antibodies. The majority of circulating lymphocytes are T cells [Eisen, 1974]. T cells can be further subdivided into the subclasses of T helper cells, T suppressor cells and cytotoxic T lymphocytes (CTL's). T helper cells facilitate immune function through the production of interleukins in response to antigen stimulation. Suppressor cells downregulate the activity of both cytotoxic T cells and T helper cells [Keast, *et al.*, 1988; Hodes, 1989].

The earliest reports of possible opioid receptors on lymphocytes came from Wybran and Govaerts [1977]. They reported that levamisole significantly increased the percentage of active and total T cells in the T cell rosette assay with sheep red blood cells (SRBC). Human T cells form rosettes with sheep red blood cells through interaction with an "E receptor" [Donahoe, *et al.*, 1985]. The rosette formation requires activated rather than resting T cells and the assay can be used to purify T cells and assess cell number.

This same group later reported that morphine reduced, while methionine-enkephalin (met-enkephalin) increased, the percentage of active T rosettes and that both of these effects were completely reversed by naloxone [Wybran, *et al.*, 1979]. The fact that morphine and methionine-enkephalin exhibited opposite effects suggested that there existed multiple opioid receptor types on lymphocytes. These initial studies led to increased interest and research in this area.

The majority of the research which investigated opioid receptors on lymphocytes utilized receptor binding assays, but many neglected to perform the necessary components of classical receptor binding studies which are necessary for identification of a physiologically relevant receptor. In classical receptor studies, receptor saturability and specificity must be demonstrated. Saturability demonstrates a finite number of receptors, which would be expected in a biological preparation. Specificity of the receptor for the ligand can be demonstrated by competition-displacement assays using other ligands known to interact with the putative receptor. Saturation is commonly analyzed through linear transformations of the data (Scatchard plots) in order to determine binding parameters, such as the dissociation constant (K_d) and the total number of binding sites (B_{max}) [Limbird, 1986].

Mehrishi and Mills [1983] reported that lymphocytes contained specific opioid receptors of the mu type as demonstrated by the binding of tritiated naloxone to freshly isolated human lymphocytes. Although this study demonstrated binding of naloxone to the lymphocytes, no saturability was demonstrated nor were competing ligands able to completely displace the bound naloxone. In contrast, Hazum *et al.* [1979] were unable

to demonstrate classical opioid receptors on lymphocytes. In this study, high affinity binding was exhibited only in a transformed human lymphocyte cell line (RPMI 6237) which was apparently of non-opioid specificity since iodinated beta-endorphin binding was not displaced by any opiate agonists, antagonists or by enkephalin analogs. Although these investigators failed to perform a saturation isotherm, they did perform a Scatchard analysis of their competition data and reported an approximate dissociation constant of 3 nM. Attempts by Mendelsohn, *et al.* [1985] to replicate these findings were unsuccessful as they were unable to demonstrate any specific binding of tritiated naloxone or tritiated ^{3}H-D-Ala^{2}-D-Leu^{5}-enkephalin to freshly isolated human lymphocytes.

Several other studies have also reported binding of various opioids and opiates to human and rat lymphocytes. Ausiello and Roda [1984] reported that tritiated leucine-enkephalin (leu-enkephalin) exhibited specific saturable binding to cultured human T lymphocytes (Jurkat lymphoid T cell line), although they were unable to inhibit the binding of leu-enkephalin with either morphine or naloxone. Although these investigators performed a saturation isotherm and reported an approximate dissociation constant (K_d), they did not discuss how their nonspecific binding was determined, nor if unlabeled leu-enkephalin was capable of displacing tritiated leu-enkephalin from the receptor. Also, the K_d was reported as a value without any units, which made interpretation of these results difficult. Madden, *et al.* [1987] also reported a specific binding site for naloxone on human T lymphocytes. They demonstrated saturable specific binding using both a saturation isotherm and competition assays and reported a K_d of 50 nM, higher than that found in neuronal tissue (1-5 nM). In this study, the bound naloxone was partially

displaced by morphine, beta-endorphin, and met- and leu-enkephalin. Another study also identified opioid binding sites on rat lymphocytes using tritiated naloxone and again reported a K_d of 10-20 nM. Binding was displaced by morphine and naloxone but not by any endogenous opioid peptides. These investigators found that spleen cells but not thymocytes exhibited specific binding to naloxone which suggested that the majority of binding seen in peripheral lymphocytes resides in the T cell population [Ovadia, *et al.*, 1989]. Another interesting fact presented by these researchers was that guanosine 5'-O-(3-thiotriphosphate) (GTP-gamma-S) inhibited the binding of naloxone. These results suggest that a GTP-binding regulatory protein that couples receptors to adenylate cyclase is involved in the binding of opiates to lymphocytes. Recently, Carr, *et al.* [1989] have reported evidence for the existence of both delta and kappa receptors on various cell lines using highly specific ligands. The existence of sigma receptors has also been reported on human peripheral blood leukocytes [Wolfe, *et al.*, 1988].

A number of recent studies support the theory that beta-endorphin binding to lymphocytes involves a non-opioid site. Westphal and Li [1984] reported that binding of tritiated beta-endorphin to neuroblastoma x glioma hybrid NG108-15 cells had a component which was not displacable by the delta ligand, [D-Ser^2]-Leu-enkephalin-Thr^6. The non-opioid binding site appeared to recognize only the carboxy terminal region of beta-endorphin. These investigators suggested that beta-endorphin recognizes two binding sites, one which is specific for the N-terminal sequence and one which is specific for the non-opioid carboxy terminal sequence. Schweigerer, *et al.* [1985b] also suggested that beta-endorphin binds to a non-opioid site on murine thymoma cells, since the binding was

not inhibited by met- or leu-enkephalin. Although these investigators did not perform a saturation isotherm, a Scatchard analysis of the competition data revealed a curvilinear plot suggesting the existence of two classes of sites, one of high affinity possessing a K_d of 65 nM and one of low affinity with a K_d of 2200 nM. Later experiments performed by this group suggested that the receptor is internalized via a high affinity site (M_r 72,000) [Schweigerer, *et al.*, 1985a]. These data are also supported by the work of Borboni, *et al.* [1989] who demonstrated specific binding of iodinated beta-endorphin to freshly isolated human lymphocytes which was not displacable by N-terminal fragments of beta-endorphin or by naloxone, morphine, bremazocine or ethylketocyclazocine. Again no saturation isotherm was performed although a Scatchard analysis of the competition data revealed a curvilinear plot. A dissociation constant was not calculated.

A receptor site containing two chains with molecular weights of 46 and 31 kd on murine splenocytes has been identified using iodinated beta-endorphin [Carr, *et al.*, 1988b]. The binding of beta-endorphin to this receptor was reversed by naloxone, suggesting that an opioid site exists to which beta-endorphin binds. Binding of beta-endorphin to this site also resulted in reduced membrane potassium conductance presumably by decreasing potassium channel openings. Beta-endorphin thus appears to act on immunocytes (T cells) as it does on neurons in the central nervous system. An antibody has also been developed which recognizes opioid receptors on murine splenocytes and neuroblastoma x glioma hybrid cells (NG108-15 cells). This antibody competes with tritiated dihydromorphine as well as beta-endorphin, met-enkephalin and naloxone for the same binding site on the lymphocytes. Binding to this receptor resulted

in suppression of cAMP production by lymphocytes by inhibiting adenylate cyclase. Inhibition of adenylate cyclase by opioid receptor interaction is also seen in neural tissue [Carr, *et al.*, 1988a]. Confusion as to the type of opioid receptor existing on lymphocytes has grown with increasing reports of conflicting data. A number of investigators report that beta-endorphin does not appear to bind to classical opiate receptors although naloxone appears to bind specifically to lymphocytes. These data suggest that there exist two types of sites on lymphocytes, "opioid" and "nonopioid". The lack of classical receptor binding studies which include saturation isotherms along with competition assays has perhaps contributed to the confusion in this area. Another factor which contributes to the confusion is the use of transformed cell lines. These cells are not representative of normal circulating lymphocytes since they differ genetically and therefore may not exhibit the same types of receptors.

2. Beta-endorphin Modulation of Immune Function

a. Proliferation Assays

The standard assay for assessing the ability of lymphocytes to respond to antigen stimulation is the lymphocyte blastogenesis assay. In this assay lymphocyte proliferation is determined by measuring tritiated thymidine uptake, as an indicator of DNA synthesis, in response to various mitogens. The mitogens most commonly used are Concanavalin A (ConA), a mitogen of both T and B cells, Phytohemagglutinin (PHA), a T cell mitogen, and Pokeweed mitogen, a B cell mitogen (although it requires the presence of T helper cells). Gilman, *et al.* [1982] reported that beta-endorphin enhanced the proliferative

response of rat splenic lymphocytes to both ConA and PHA in a dose dependent manner. Alpha-endorphin and [D-Ala^2, Met^5]enkephalin did not affect the proliferative responses and naloxone was unable to reverse the enhancement produced by beta-endorphin. Kusnecov, *et al.* [1989] also reported that beta-endorphin enhanced ConA-stimulated rat splenocyte proliferation with no stimulation by alpha-endorphin or methionine-enkephalin, but they did not attempt to reverse the beta-endorphin enhancement with naloxone. Both of these investigators concluded that beta-endorphin acts through a non-opioid site to influence cell proliferation. Beta-endorphin also appeared to enhance murine lymphocyte proliferation in response to ConA with the carboxy terminal portion of the molecule required for activity. Again, it was concluded that beta-endorphin interacts with a non-opioid receptor [Gilmore and Weiner, 1989].

Reports of beta-endorphin modulation of human lymphocyte proliferation generally indicate suppression of the mitogenic response. McCain, *et al.* [1982] showed that beta-endorphin suppressed PHA stimulated blastogenesis and this suppression was not blocked by naloxone. The results were supported by Puppo, *et al.* [1985] who also reported suppression of PHA-induced lymphocyte proliferation which was not reversed by naloxone. In contrast, Heijnen, *et al.* [1987] demonstrated that beta-endorphin was capable of either enhancing or inhibiting ConA induced proliferation. The ability of beta-endorphin to either enhance or suppress proliferation depended on both concentration and also donor. The activity seems to reside in the carboxy terminal sequence since removal of the N-terminal did not affect activity. This work was supported by McCain, *et al.* [1987] who demonstrated that the carboxy terminal dipeptide of beta-endorphin, glycyl-L-

glutamine, enhanced the response of lymphocytes to PHA at low doses (10^{-12} M) and suppressed blastogenesis at higher doses (10^{-7} M). These studies suggested that beta-endorphin is capable of both suppressing and enhancing lymphocyte blastogenesis, but that the effect may be dependent on the mitogen used, the dose, the individual and the species.

b. Natural Killer Cell Activity

Natural killer cells are a subpopulation of T lymphocytes that spontaneously recognize and lyse tumor and virally infected cells and are therefore thought to be critical in the immediate host defense to viral infections and the rejection of malignant cells. Opioid forms of stress (i.e. intermittent inescapable footshock stress) suppress the cytotoxic activity of natural killer (NK) cells as do daily injections of morphine in the rat [Shavit, *et al.*, 1984]. Methionine- and leucine-enkephalin both enhanced NK cell activity in peripheral blood lymphocytes from cancer patients at a concentration range from 10^{-14} to 10^{-6} mg/ml [Faith, *et al.*, 1987]. In contrast, Oleson and Johnson [1988] reported that the enkephalins enhanced NK activity in low responder populations (basal activity of less than or equal to 50 lytic units) and suppressed in high responder populations (basal activity greater that 50 lytic units). In was concluded, therefore, that naloxone displayed both antagonist and agonist properties in this study.

Beta-endorphin has been reported to augment NK cytotoxicity by a number of investigators. Mathews, *et al.* [1983] and Kay, *et al.* [1984] both reported that beta-endorphin enhanced NK cell cytotoxic activity in a dose-dependent and naloxone-

reversible manner. In another study, beta-endorphin also augmented NK cytolytic activity by 63% in a naloxone-reversible manner, whereas neither alpha- or gamma-endorphin were able to augment activity [Mandler, *et al.*, 1986]. Kay, *et al.* [1987] demonstrated enhancement of NK cytotoxicity by non-opioid fragments of beta-endorphin, i.e. those lacking the N-terminal tyrosine. These fragments were more potent than beta-endorphin and the effect was reversed by naloxone. Consequently, two receptors sites may be present on NK cells: one activated by opioids, another by certain non-opioid fragments. Also, since naloxone could reduce the non-opioid activity, there may be some type of interaction between these two receptor sites. It has also been reported that beta-endorphin and methionine-enkephalin can enhance the generation of cytotoxic T cells and this enhancement can be partially blocked by naloxone. Simultaneous addition of beta-endorphin and met-enkephalin did not increase the enhancement of cytotoxic T lymphocytes over that of either peptide alone, therefore suggesting that they were acting at the same receptor [Carr and Klimpel, 1986].

Although the majority of the studies reported enhancement of NK cell function by beta-endorphin, one study did report that beta-endorphin suppressed NK cell activity by 50% and that this effect was reversed by naloxone and was not due to changes in T cell subsets [Prete, *et al.*, 1986]. These investigators used a different experimental design where beta-endorphin was incubated with the lymphocytes prior to the actual cytolytic assay rather than throughout the assay as was done in the previously mentioned studies. They concluded that beta-endorphin suppressed NK cell activity through interaction with classical opioid receptors and that lymphocytes may possess both opioid and non-opioid

receptors which may explain the discrepancy between their work and others. McCain, *et al.* [1986] reported that beta-endorphin induced T cell suppressor activity and therefore may suppress immune function through activation of specific suppressor T cell populations. It seems therefore that beta-endorphin is capable of both enhancing and suppressing natural killer cell activity. This discrepancy may be explained by: 1) the existence of two populations of NK cells, i.e. low and high responders, 2) the possibility that NK cells possess both opioid and non-opioid receptors with which beta-endorphin is capable of interacting, or 3) that beta-endorphin may be enhancing T cell suppressor activity.

c. Macrophage and Granulocyte Activity

Beta-endorphin has been reported to modulate granulocyte and macrophage function in a number of ways. Opioid peptides have been reported to both enhance and inhibit granulocyte chemotaxis. Simpkins, *et al.* [1984] demonstrated a 20% increase in migration of granulocytes by beta-endorphin toward the attractant N-formyl methionyl leucyl phenylalanine (FMLP) in a soft agar medium, which was antagonized by naloxone. In contrast, Marcoli, *et al.* [1988] reported that both an enkephalin analog, dynorphin (1-9) and morphine inhibited casein-stimulated chemotaxis. In this assay, naloxone appeared to have an inhibitory action of its own. The enkephalin analog and dynorphin had no effect on spontaneous migration while morphine was able to increase spontaneous migration. Beta-endorphin has also been shown to enhance chemotaxis of human monocytes in a stereospecific manner, as (-)naloxone was capable of reversing this effect

while (+)naloxone was not [Ruff, *et al.*, 1985]. Beta-endorphin appeared to suppress production of the T lymphocyte chemotactic factor in a bimodal fashion with peaks of activity occurring at 10^{-11} M and 10^{-6} M [Brown and Van Epps, 1985].

Human neutrophil adherence was increased by both beta-endorphin and met-enkephalin in a concentration dependent manner and this effect was partially reversed by naloxone [Van Epps and Kutvirt, 1987]. Again, the response appeared to be bimodal with the greatest peaks of activity occurring at 10^{-13} to 10^{-12} M and 10^{-8} to 10^{-9} M. Falke and Fischer [1985] demonstrated changes in polymorphonuclear leukocyte cell shape caused by beta-endorphin. Beta-endorphin induced both cell spreading and elongation in a manner equivalent to that of the chemotactic agent FMLP, and these changes could be antagonized by the opiate antagonist diprenorphine.

Beta-endorphin stimulates superoxide production in both neutrophils and macrophages. Superoxide production and the generation of other reactive oxygen species is linked to microbicidal activity and the regulation of natural killer cell activity by polymorphonuclear cells and monocytes. Beta-endorphin and dynorphin stimulate superoxide production in granulocytes and macrophages. The effect is rapid and sustained in granulocytes but brief in macrophages. Naloxone inhibits the response by 75% in both cell types [Sharp, *et al.*, 1985]. This same group went on to demonstrate that the stimulation of superoxide production of neutrophils by beta-endorphin was stereoselective and apparently via an opioid receptor, since N-acetyl-beta-endorphin and (+)naloxone had no effect [Sharp, *et al.*, 1987]. Although beta-endorphin appears to stimulate superoxide production in neutrophils, met-enkephalin and leu-enkephalin have been shown to inhibit

superoxide release by neutrophils stimulated with FMLP [Simpkins, *et al.*, 1986].

d. Antibody and Interferon Production

Beta-endorphin has been shown to influence *in vitro* antibody and interferon production. Alpha-endorphin, met-enkephalin, leu-enkephalin and beta-endorphin suppressed the *in vitro* antibody production of mouse spleen cells. Alpha-endorphin, which contains the first sixteen N-terminal amino acid residues of beta-endorphin, was a potent suppressor while beta-endorphin only minimally inhibited antibody production [Johnson, *et al.*, 1982]. Heijnen, *et al.* [1986] also demonstrated suppression of antibody production by alpha-endorphin in human lymphocytes. The inhibition of antibody formation by alpha-endorphin occurred both at the T and B cell level. Removal of the N-terminal tyrosine resulted in loss of activity, therefore suggesting that alpha-endorphin inhibition of antibody production resulted from interaction with an opioid receptor. Most recently, Williamson, *et al.* [1988] demonstrated a biphasic effect of beta-endorphin on the production of specific anti-herpes viral antibodies *in vitro*. At concentrations of 10^{-14} M and below, beta-endorphin enhanced antibody production, while at higher concentrations suppression of the antibody response occurred. It was noted that the effects of beta-endorphin on human natural killer cells are also biphasic but are a mirror image of the effects on antibody production, suggesting that the immunomodulatory effects of beta-endorphin may be mediated by effects on natural killer suppressor-inducer cells.

In addition to modulating antibody production, beta-endorphin enhanced

interleukin-2 production in mitogen stimulated murine lymphocytes. The enhancement was not naloxone reversible and was dependent on the presence of the carboxy-terminal amino acids although the N-terminal amino acids contributed to the potency [Gilmore and Weiner, 1988]. Beta-endorphin also modulated the production of gamma-interferon by peripheral blood mononuclear cells. Brown and Van Epps [1986] demonstrated enhanced interferon production in Con A-stimulated mononuclear cells by beta-endorphin which was not prevented by naloxone. Peterson, *et al.* [1987] reported conflicting results, in which beta-endorphin inhibited gamma-interferon production by Con A-stimulated mononuclear cells. In this study, the suppression was antagonized by naloxone and was specific for the N-terminal of beta-endorphin, suggesting that a classical opioid receptor was involved. Also, monocytes appeared to be the primary target cells and reactive oxygen intermediates (ROI) were important mediators in that scavengers of ROI eliminated the suppression.

3. Opioid Peptide Production by Cells of the Immune System

The hypothesis that the immune and neuroendocrine systems interact and modulate one another is supported by research that indicates that cells of the immune system synthesize opioid peptides which are biologically active. Immunoreactive pro-enkephalin A activity, as well as proenkephalin A-like RNA, has been identified in human leukocytes from patients with chronic lymphoblastic leukemia [Monstein, *et al.*, 1986]. This work was confirmed by Zurawski, *et al.* [1986], who demonstrated preproenkephalin messenger RNA in a Con A-activated mouse T-helper cell line. Induced T-helper cell culture

supernatants also demonstrated met-enkephalin immunoreactive material. Using T helper cell lines, Roth, *et al.* [1989] showed that posttranslational processing of the precursor, proenkephalin, is different in lymphocytes than in the brain or the adrenal gland. They found that activated T helper cells secreted significant concentrations of high molecular weight, opiate-inactive peptides which differed from those derived from the neuroendocrine system.

Beta-endorphin and related proopiomelanocortin derived-peptides have been identified in cells of the immune system. Mouse spleen macrophages have been shown to contain immunoreactive beta-endorphin and ACTH [Lolait, *et al.*, 1984]. Infection of human lymphocytes with Newcastle disease virus stimulated production of immunoreactive beta-endorphin that is identical to pituitary beta-endorphin, based on antigenicity, molecular size, association with ACTH and biologic activity [Smith, *et al.*, 1985]. Bacterial lipopolysaccharide also induced *de novo* synthesis of immunoreactive ACTH and alpha- and gamma-endorphin [Harbour-McMenamin, *et al.*, 1985]. The production of ACTH and endorphins by leukocytes is induced by corticotropin releasing factor (CRF) and suppressed by dexamethasone in a manner similar to that of pituitary cells [Smith, *et al.*, 1986]. The opioid gene for proopiomelanocortin (POMC) has been identified in Newcastle disease virus-infected murine splenocytes which offers more evidence that lymphoid cells can be activated to express POMC or its products [Westly, *et al.*, 1986].

There appear to be two classes of stimulants for ACTH and endorphin production by leukocytes. The first is immunostimulants which include viruses, tumor cells and

bacterial lipopolysaccharide. The other class is the hypothalamic-pituitary peptides, such as corticotropin releasing factor. Leukocytes therefore appear to be similar to pituitary cells in that the POMC gene is controlled by signals from the hypothalamus and feedback inhibition by corticosteroids. Although these similarities exist, leukocytes appear to differ somewhat from pituitary cells in posttranslational processing. Whereas Newcastle disease virus and CRF cause the production of ACTH and beta-endorphin, bacterial lipopolysaccharide elicits the production of immunoreactive ACTH and endorphins which correspond to the molecular weights of alpha- and gamma-endorphin [Blalock, 1985].

In summary, although the evidence suggests that leukocytes possess opioid receptors, the presence of these receptors on lymphoid cells remains poorly characterized. The lack of classical binding techniques has led to a number of confusing results of which some can not be interpreted. The evidence for these receptors led researchers to investigate a possible role for endogenous opioids in immune function. Beta-endorphin appears to modulate a number of immunological parameters although the exact nature of the effect often appears to depend on the cell type, species involved, the individual and the dose used. The role of beta-endorphin becomes more complicated when the opioid and non-opioid effects are considered. The presence of the POMC gene, immunoreactive ACTH and beta-endorphin in leukocytes lends further credence to the role of beta-endorphin as an immunomodulator and suggests that leukocyte-derived beta-endorphin may autoregulate and/or modulate immune function.

D. EXERCISE AND IMMUNE FUNCTION

Evidence is accumulating which supports the theory that physical exercise, as a form of exertional stress, is capable of modifying immune function. Physical exercise over a wide range of exertion levels causes a marked increase in the number of circulating leukocytes in humans. The number increased within 5 minutes of beginning submaximal exercise and remained elevated for at least 15 minutes after cessation of exercise. The degree of leukocytosis may be inversely proportional to the amount of previous training [Keast, *et al.*, 1988].

Physical exercise may also modify the proportions of lymphocyte subsets. Hedfors, *et al.* [1976] reported that short term exercise resulted in increased lymphocyte number in humans with decreased T lymphocytes, increased B lymphocytes, increased NK-cell cytotoxicity, and impaired response to the mitogens Con A, PHA and PWM. Later work by this group demonstrated decreased T lymphocytes after exercise which appeared to be due to a reduction of T helper cells. Results from this study also showed reduced antibody production and reduced lymphocyte DNA synthesis after stimulation by allogeneic cells [Hedfors, *et al.*, 1983]. Exercise consisting of 5 minutes of running up and down stairs resulted in an increase in cytotoxic/suppressor lymphocytes and NK cells. The increase in NK cells was associated with significantly greater NK cell activity but there was no change in the proliferative response to the mitogen Con A [Edwards, *et al.*, 1984]. Acute exhaustive treadmill exercise also resulted in a decreased number of T helper cells and an increased number of T suppressor cells with no difference between athletes and non-athletes [Berk, *et al.*, 1985]. Although the previously mentioned studies

reported a decreased ratio of helper to suppressor cells, Bongers and Bertrams [1984] were unable to find any differences in T cell subsets following exercise.

In general, most levels of exercise stress produce a transient suppression of lymphocyte transformation in response to mitogens. As mentioned before, Hedfors, *et al.* [1976,1983] demonstrated decreased responsiveness to mitogens following exercise. Eskola, *et al.* [1978] also reported depressed responsiveness to the mitogens PHA and Con A after 2.5 hours of marathon running but were unable to detect any change in lymphocyte transformation following 35 minutes of running. In contrast, Edwards, *et al.* [1984] were unable to detect any change in T cell proliferation following exercise. The previous work was performed exclusively in humans. One study in horses demonstrated a significant suppression of the blastogenic response to the mitogens Con A and PHA, 30 minutes after treadmill exercise to fatigue [Kurcz, *et al.*, 1988].

The ability of exercise to modify the humoral immune response is not as clearcut. Hedfors, *et al.* [1983] reported suppressed antibody production following exercise while Eskola, *et al.* [1978] were unable to demonstrate any change in antibody production following a marathon. There are a number of other conflicting studies. Reductions of both serum and secretory immunoglobulins have been linked to increasing intensity of exercise in humans, while in mice and rats no difference could be detected in the response of antibody production to antigen inoculation in exercised animals [Keast, *et al.*, 1988].

Several investigators have reported that exercise results in an increased number of NK cells and enhanced activity [Keast, *et al.*, 1988]. One particularly interesting study demonstrated enhanced NK cell activity after a maximal bicycle ergometer exercise test

which was antagonized by naloxone administration prior to exercise. Significant stimulation of NK cell activity was observed with beta-endorphin *in vitro* before exercise but not after. When naloxone was given prior to exercise, exercise no longer blocked the *in vitro* stimulation of NK cells by beta-endorphin [Fiatarone, *et al.*, 1988]. These results suggest that the opioid system plays a role in the modulation of NK cell function following exertional stress. Enhancement of NK cell activity following exercise is of interest in that there have been reports of decreased tumor growth in exercised animals [Keast, *et al.*, 1988].

Exercise has also been reported to modulate the function and number of phagocytic cells. Exhaustive exercise has been found to decrease the binding efficiency of insulin to monocytes while moderate exercise increased the binding affinity. The number of insulin receptors on monocytes has been found to increase over a physical training period suggesting an increase in the phagocytic activity of the cells. Neutrophil function also decreased following exercise [Keast, *et al.*, 1988].

Although most studies appear to indicate increased leukocyte number and temporary immune suppression following exercise, there are conflicting results due perhaps to the large variation in exercise protocols with respect to exercise duration and intensity. Although exercise appears to suppress lymphocyte proliferation and phagocyte function, exercise is reported to augment NK cell function and therefore perhaps suppress tumor growth. The enhancement of NK cell function is perhaps a result of changes in opioid peptide levels following exercise since administration of naloxone prior to exercise blocks the increase in natural killer cell activity. Several studies also suggest that

exercise results in changes in lymphocyte subsets, i.e. decreased T helper cells and increased T suppressor cells. The decreased helper/suppressor cell ratio following exercise may be responsible for the temporary immune suppression which follows certain types of exercise. These results suggest that overtraining may increase susceptibility to infection.

E. ANALYSIS OF RADIOLIGAND BINDING DATA

Radioligand binding assays allow direct identification of binding sites in target tissues by studying the binding of a radiolabeled drug or hormone to a receptor. In order to measure receptor binding, the assay requires a radioactively labeled drug or hormone, a tissue receptor preparation and a method for separating bound from free drug or hormone. The reaction of the drug or hormone with the receptor is said to follow the law of mass action if there is a single ligand reacting with a single population of homogenous binding sites by a fully reversible reaction. The law of mass action is described by the following equation:

$$[D] + [R] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [DR]$$

where [D] = hormone or drug

[R] = receptor

[DR] = complex of the receptor with drug

k_1 = association rate constant

k_2 = dissociation rate constant

The choice of isotope for the radiolabeling of the ligand is a critical consideration in that full biological activity is necessary for accurate measurement of binding to the receptor. Tritium is popular because it has a long half-life but it is limited in its ability to detect receptors with limited availability because of its relatively low specific activity (29.4 Ci/mmol). In contrast, iodinated ligands, because they possess high specific activity (2200 Ci/mmol) and do not significantly alter biological activity, are frequently used when receptors are low in quantity and/or possess high affinity for the ligand. Optimum incubation conditions must be determined empirically for each tissue preparation used, such as intact cells, homogenates or isolated membrane fractions. The method used to separate bound from free ligand is also critical in that the separation process must not disturb equilibrium conditions [Limbird, 1986].

There are certain minimal criteria which must be met in order to establish binding to a physiologically relevant receptor. The binding of the ligand to the receptor should be saturable since a finite number of receptors are expected in a biological preparation. The binding should also be specific. This can be demonstrated by showing competition between the radioligand and other ligands known to interact with the putative receptor. The specificity of these ligands at the receptor should parallel the specificity of the ligands *in vivo*. Finally, the kinetics of the binding should be consistent with the time-course of the biological effect elicited by the ligand.

The saturability of the receptor is usually assessed by performing a saturation isotherm, in which the characteristics of binding as a function of increasing concentrations of the radioligand are determined. When the bound radioactivity [*DR] represents

saturable binding to a receptor possessing a single affinity, K_d, for the radioligand [*D], the plot of [*DR] versus [*D] will yield a rectangular hyperbola (Figure 3A). It is important to note that K_d (the dissociation constant), a measure of the affinity of the receptor for the ligand, is the concentration of the ligand that half-maximally occupies the receptor. Also, when a saturation isotherm is plotted as [*DR] versus [*D] resulting in a rectangular hyperbola, the horizontal asymptote is B_{max} or the total receptor concentration or density. Therefore B_{max} will be attained only at infinite concentrations of the radiolabeled ligand. Not all of the radioligand binding is necessarily so-called "specific" binding. Some radioactivity which is measured as bound ligand is actually "bound" or trapped to other sites, such as in the pellet or nonspecific binding to the membrane. Consequently, a valid determination of nonspecific binding is very important in setting up a reliable assay. Nonspecific binding increases linearly as a function of increasing concentrations of [*D] and therefore would not be saturable. Nonspecific binding can be determined by binding which cannot be competed for by unlabeled ligand present at 100 x K_d of the unlabeled ligand. Nonspecific binding can also be defined using computer-assisted mathematics. Specific binding is traditionally calculated by subtracting nonspecific binding from total binding [Limbird, 1986].

Saturation binding data can be plotted two different ways. It can be plotted on a linear scale of [*DR] versus [*D] (Figure 3A) or it can plotted as [*DR] versus Log_{10} [*D] (Figure 3B). On a linear scale the specific binding appears to plateau, giving the impression that a rectangular hyperbola or saturation of the receptor has been obtained. Transformation of the data with a plot using a log scale on the x-axis reveals a truncation

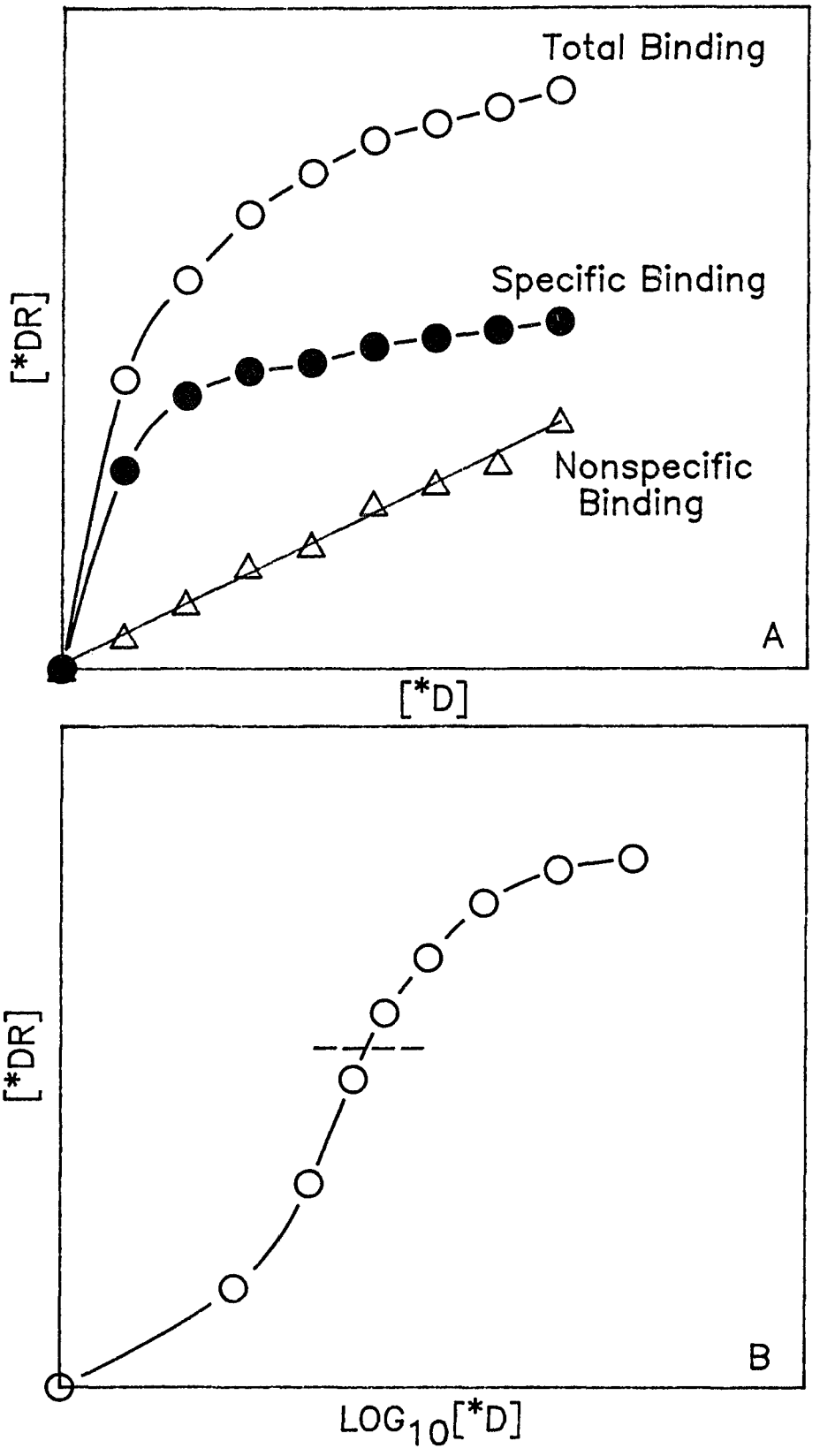

Figure 3. Linear and log saturation isotherms of radioligand binding data.

of the isotherm which occurs when too narrow a range of radioligand concentrations are used. Unfortunately, cost considerations and a second steeper slope of nonspecific binding may preclude saturation isotherms which even approach actual saturation of the receptor population. Therefore, linear transformations of binding data have been developed which allow estimation of the affinity of the receptor-ligand interaction (K_d) and the receptor density (B_{max}) [Limbird, 1986].

There are several linear transformations which can be used to analyze radioligand binding data. Linear transformations give the advantage of estimating various parameters from the slope and the x- and y-intercept, all of which can be easily determined. However, linear transformations tend to distort the data, especially if extrapolated from data obtained over an insufficient range of concentrations of radioligand, and therefore may not disclose the true complexities of the receptor-ligand interaction. The most commonly used transformation is the Scatchard plot in which receptor affinity can be estimated from the slope of the plot and receptor density from the x-intercept. Another linear transformation is the Hill plot in which the K_d can be estimated from the x-intercept. The slope is referred to as the Hill coefficient, which can be used to determine the complexity of the ligand-receptor interaction. Least used is the double-reciprocal or Lineweaver-Burk plot. This plot, of all the linear transformations, results in the greatest distortion of the data and therefore results in limited or uncertain extrapolations [Limbird, 1986].

As the Scatchard plot is the most frequently used linear transformation of radioligand binding data, a detailed discussion of its uses and limitations follows. The

Scatchard plot is a plot of [*DR]/[*D] on the ordinate versus [*DR] on the abscissa or B/F versus B (Figure 4A) where B or [*DR] is the concentration of radioligand present as a ligand-receptor complex at equilibrium and F or [*D] is the concentration of free radioligand present at equilibrium. From this plot, the data is transformed to a linear expression of $y = mx + b$ or $B/F = -1/K_d B + B_{max}/K_d$, where m is equal to the slope or $-1/K_d$ and b is equal to the x-intercept or B_{max}. In a genuine Scatchard plot the concentration of the receptor species is known and therefore the x-intercept would give the number of binding sites per mole of receptor, not the total density of binding sites [Scatchard, 1949]. Consequently, a modification of the Scatchard plot, the Rosenthal plot, is used. The Rosenthal plot assumes that the concentration of receptors is unknown and therefore the x-intercept is a determination of the total receptor concentration ($[R]_{TOT}$) only when one population of binding sites exists; that is, when $n = 1$, $n[R]_{TOT}$ estimates $[R]_{TOT}$. Otherwise the x-intercept determines the total number of binding sites rather than total receptor concentration [Limbird, 1986; Rosenthal, 1967].

A number of assumptions are made in the algebraic derivations for the linear transformations which have been described. If the assumptions are not met when obtaining the raw data, then the interpretation of the Scatchard plot will be invalid. The first assumption is that the ligand-receptor interaction follows the law of mass action; that is, a single drug is interacting with a single receptor population in a fully reversible manner. If this requirement is not met, a single slope will not be obtained. The second assumption is that the binding must have attained equilibrium. Another assumption is that bound and free radioligand can be determined accurately. Therefore, an error in the

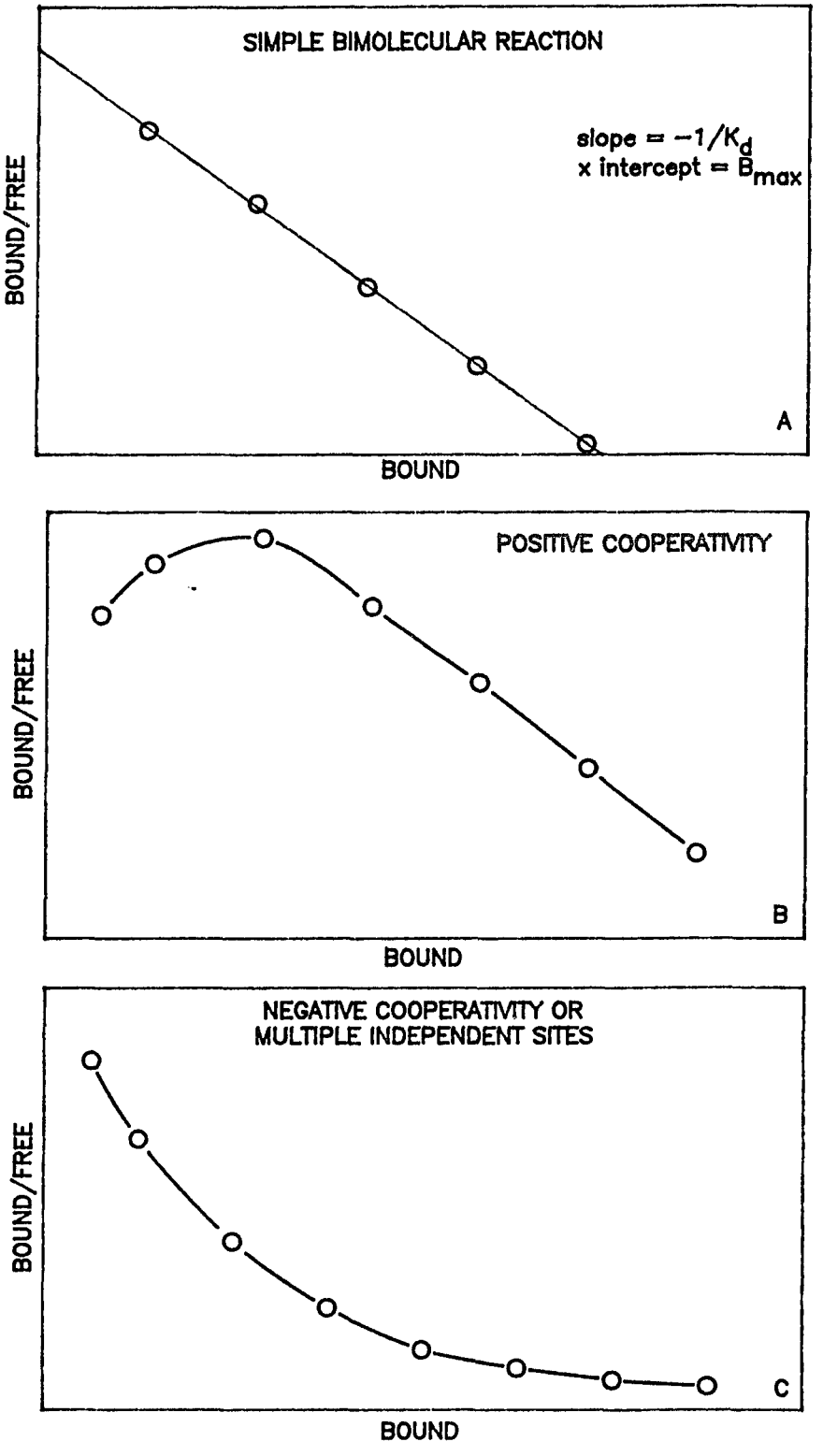

Figure 4. Sample Scatchard plots representing a simple bimolecular reaction (A), positive cooperativity (B), and negative cooperativity (C).

determination of nonspecific binding will introduce artifactual error into the binding parameters obtained from a Scatchard analysis [Limbird, 1986].

A linear Scatchard plot allows for correct estimation of K_d and B_{max} from the slope and x-intercept. However, frequently nonlinear or curvilinear Scatchard plots are observed which makes interpretation of the plot difficult and confusing as well as controversial. Nonlinear Scatchard plots can be concave downward or concave upward. A concave downward Scatchard plot (Figure 4B) may be interpreted to result from positive cooperativity of the receptor population. Positive cooperativity results when the affinity of the overall receptor population increases with increasing receptor occupancy. Positive cooperativity may result from receptor-receptor interactions within multisubunit receptor complexes or between physically separate receptors or from the binding of a ligand to a single receptor which possesses multiple binding sites and whose affinities are increased by sequential binding to the receptor. Downward concave Scatchard plots can also result from artifactual sources such as: determination of [*DR] before equilibrium is reached, inactivation of the ligand during incubation, inactivation of the receptor during incubation and stabilization of the receptor against degradation during occupancy by the ligand. A concave upward Scatchard plot (Figure 4C) may result from multiple orders of noninteracting binding sites, existence of multiple affinity states of the receptor or negative cooperativity. Technical problems which lead to artifactual upward concave Scatchard plots are: inappropriate definition of nonspecific binding, aggregation of the ligand at higher concentrations to a dimer or multimer which has lower affinity for the receptor and higher affinity of the radiolabeled ligand than the unlabeled ligand for the

receptor [Limbird, 1986].

The popularization of the Scatchard graph has led to its use without a true understanding of its limitations. This trend has led to some controversy as to the legitimacy of its use in a number of situations. Klotz [1982] suggested that the extrapolation necessary to calculate the total number of receptors is generally not correct unless a plot of [B] versus log [F] is done in order to determine if the data has been obtained over a sufficiently broad concentration range of the radioligand. He concluded that the Scatchard plot is, in principle, correct only when extensive measurements are made and only the simplest ligand-receptor interaction is occurring. Munson and Rodbard [1983] also noted that the true value of B_{max} can never be proven through a Scatchard analysis and one can only make an estimate of B_{max} based on a particular model, such as one homogenous site or two or three classes of independent sites. They noted that small errors in data are magnified at either axis of a Scatchard plot but that similar magnification of error is also seen in the upper plateau when [B] is plotted against log [F]. These investigators suggest apparent K_d and B_{max} values can be legitimately used under defined experimental conditions in order to determine changes as a result of a "treatment" as opposed to a "control".

The development of computerized least-squares curve fitting which is weighted to account for the statistical distributions of the errors may enable the investigator to avoid graphical methods altogether. An example is the computer program "LIGAND" which provides weighted least squares estimates of binding parameters such as affinity constants, binding capacities and nonspecific binding [Munson and Rodbard, 1980].

However, the mathematical model upon which the computer program is based may not necessarily describe the molecular model which accounts for the ligand-receptor interaction and therefore may limit the validity of the computer-derived estimates of binding parameters [Limbird, 1986].

Competitive binding analysis is usually performed in order to determine the specificity of the radioligand binding. Due to technical and cost considerations, many investigators perform Scatchard analysis of competitive binding data rather than saturation isotherms to determine binding parameters. Data from competitive binding studies are usually plotted as the percent specific binding versus the Log_{10} [competitor]. Whether or not a competitor is interacting with the receptor by simple mass action law or with greater complexity, such as multiple affinity states, can be determined by the overall "shape" of the competition binding curve. If the ligand-receptor interaction is a simple bimolecular reaction, the competition curve for the competitor X will proceed from 10% to 90% competition over an 81-fold concentration range of X. Therefore curves which do not exhibit "normal steepness" are said to result from complex ligand-receptor interactions [Limbird, 1986]. Many investigators mistakenly report the ligand concentration at which 50% of the bound radioligand is displaced as the dissociation constant of the ligand. However, the dissociation constant derived this way actually exceeds the actual dissociation constant, particularly if it is measured at high labeled ligand or binding site concentrations [Jacobs, *et al.*, 1975]. The binding dissociation constant can be calculated from competitive displacement assays if a radiolabeled compound with a known K_d is used [Linden, 1982]. However, the calculation is a long, tedious process which does not

lend itself to routine use. Therefore, many investigators perform Scatchard analyses of their competition binding data. The use of the Scatchard analysis for competitive displacement analysis of binding parameters is thought to be valid if the ligand-receptor interaction is simple competitive inhibition [Weiland and Molinoff, 1981]. However, artifactually nonlinear Scatchard plots result if the labeled and unlabeled ligands do not bind to the receptor with equal affinity. If the radiolabeled ligand binds to the receptor with lower affinity than the unlabeled ligand, a concave downward Scatchard plot will result. Conversely, if the radioligand binds with higher affinity to the receptor than the unlabeled ligand, then a concave upward Scatchard plot will be seen [Taylor, 1975].

In summary, radioligand binding assays provide a means for direct identification of biological receptors. In order to characterize a physiologically relevant receptor, it is necessary to show saturability, specificity and kinetics which parallel the time course of the biological effect. Scatchard analysis of radioligand binding data enables estimation of the dissociation constant (K_d) and the total number of binding sites (B_{max}). However, a good understanding of the limitations of the Scatchard plot are necessary for valid interpretation of radioligand binding data. Technical and cost limitations have resulted in the use of Scatchard analysis of competitive displacement studies. Although theoretically this is a valid use of this graphical interpretation of radioligand data, no comparisons have been made between Scatchard analysis of saturation binding data and competitive binding data using the same ligands. Therefore, Scatchard analysis of radioligand binding data requires proper knowledge of the assumptions upon which the

algebraic derivations are made and accurate measurements in order to insure that interpretations are valid. It is also important to note that the parameters derived from this form of analysis are estimates only, as the values obtained result from extrapolations of the data and every investigators' experimental conditions differ.

CHAPTER THREE

DIURNAL VARIATION IN BETA-ENDORPHIN LEVELS AND NOCICEPTIVE THRESHOLDS IN THE HORSE

A. INTRODUCTION

Diurnal variation in both plasma and cerebrospinal fluid levels of beta-endorphin has been demonstrated in humans and monkeys with the highest levels occurring around 0800 hours [Petraglia, *et al.*, 1986; Farsang, *et al.*, 1983; Barreca, *et al.*, 1986; Naber, *et al.*, 1981]. A diurnal rhythm of the precursor proopiomelanocortin messenger RNA has also been demonstrated in the intermediate pituitary of rats. The variation of the proopiomelanocortin (POMC) messenger RNA parallels that of beta-endorphin and these events are preceded by similar changes in POMC gene transcription [Millington, *et al.*, 1986].

Decreased pain sensitivity at night has been demonstrated in the rat and mouse [Wright, 1981; Frederickson, *et al.*, 1977]. This diurnal rhythm in nociceptive thresholds coincides with the diurnal rhythm of endogenous opioids, since both the rat and mouse are nocturnal animals with higher opioid levels occurring at night [Tang, *et al.*, 1984]. Wesche and Frederickson [1979] have correlated diurnal variation of opioid peptide levels with nociceptive sensitivity in the mouse, although McGivern and Bernts.n [1980] suggested that the analgesic response may be due to patterns of food intake rather than circadian rhythm. Wesche and Frederickson [1979] also demonstrated higher levels of

endogenous opioid peptides after a noxious stimulus in the afternoon as opposed to the morning in mice, suggesting that the endogenous opioid system exhibits tonic activity. In this study we present evidence of a diurnal rhythm in both pain sensitivity and plasma beta-endorphin levels in the horse.

B. MATERIALS AND METHODS

Eight mature Thoroughbred horses (seven mares; one gelding) were used. All subjects were acclimated to their stalls 24 hours before each experiment. Nociceptive thresholds, plasma beta-endorphin and cortisol levels and physiological parameters were measured at different times of the day: 0600, 0900, 1200, 1500, 1800, and 2400 hours. Experiments at each of the times were performed at least 4 days apart to avoid conditioning. Nociceptive thresholds were measured using the method of Kamerling, *et al.* [1985b]. A beam of intense light from a radiant-heat lamp[1] was focused from a fixed distance on the withers, then the fetlock. The elapsed time from turning on the lamp to cutaneous musculature contraction was called the skin-twitch reflex latency (STRL); the elapsed time to limb withdrawal was called the hoof-withdrawal reflex latency (HWRL). The area of skin on both the withers and fetlock was shaved and blackened with india ink to provide a uniform surface for heat absorption. Lamp intensity was standardized by maintaining lamp voltage at 80 V to produce a reaction within 5 to 10 seconds. Voltage was calibrated at every use. A cut-off latency of 15 seconds was chosen to prevent tissue damage.

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[1]Custom design.

Cardiac rate was measured from polygraph recordings[2] of an electrocardiogram. Respiratory rate was determined from polygraph recordings[3] using impedance pneumography. Vertical pupil diameter was obtained from photographs of the eye after standardizing the distance from camera[4] to eye. Rectal temperature was recorded from a deep rectal probe[5] and a digital thermometer[6].

Blood samples for determination of beta-endorphin and cortisol levels were taken in the stall immediately prior to other measurements. Blood samples were obtained from the jugular vein into 7 ml evacuated siliconized glass tubes containing EDTA as an anticoagulant. The samples were immediately centrifuged, plasma removed and frozen at -20°C until the assays were performed. Plasma beta-endorphin levels were measured using a commercially available radioimmunoassay (RIA) kit[7] for use in humans. This RIA kit consisted of two procedures. The first procedure involved the extraction of beta-endorphin from plasma using specific adsorption particles, i.e. affinity gel extraction. Extraction of beta-endorphin from plasma was necessary because iodinated beta-endorphin tends to nonspecifically bind to high molecular weight plasma components at an alkaline

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[2]Grass Polygraph, Model 7, EKG Tachograph preamplifier, Model 7P4F, Grass Instruments, Quincy, Mass.

[3]Grass Polygraph, Model 7, Low-level DC pre-amplifier, Model 7P1FG, Grass Instruments, Quincy, Mass.

[4]Polaroid CU-5 close-up camera, Polaroid Corp., Cambridge, Mass.

[5]Rectal probe, Model RET-1, Sensortek, Clifton, N.J.

[6]Digital thermometer, Model TH-6D, Sensortek, Clifton, N.J.

[7]Plasma beta-endorphin RIA, INCSTAR Corporation, Stillwater, Minn.

pH [Orf, *et al.*, 1979]. This binding interferes with the RIA and makes either extraction or column chromatography necessary. Neither silicic acid nor SP-Sephadex adequately separate beta-endorphin from the plasma components, therefore affinity gel extraction is used as an effective method of concentrating beta-endorphin. The second procedure is the RIA itself, which was a disequilibrium method based on an antibody with high sensitivity to beta-endorphin. The sample or standard was incubated with the rabbit anti-beta-endorphin serum for 16-24 hours at 2-8°C. Iodinated beta-endorphin was then added and a second incubation of 16-24 hours at 2-8°C followed. Separation of the beta-endorphin bound to the first antibody was achieved by addition of the goat anti-rabbit precipitating complex, a short incubation of 15-25 minutes at 2-8°C followed by centrifugation at 760 X g for 20 minutes. The supernatant was then decanted and the pellet was counted for two minutes using a gamma scintillation counter[8]. This kit was validated for use in horses by determining specificity, accuracy and precision in the measurement of equine beta-endorphin[9].

Plasma cortisol levels were also measured by a commercially available RIA kit[10]. This procedure was a solid-phase RIA in which the antibody was attached to the wall of a polypropylene tube. The sample or standards were added to the tubes, iodinated cortisol was then added and the tubes were incubated for 45 minutes at 37°C. The tubes were then decanted and counted for one minute on a gamma scintillation counter. The kit was

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[8]Searle Automatic Gamma System, Model 1185, Searle Analytic Inc., Des Plaines, Il.

[9]Equine beta-endorphin, Dr. C.H. Li, Hormone Research Laboratory, University of California, San Francisco, Calif.

[10]Cortisol Coat-a-Count RIA, Diagnostic Products Corporation, Los Angeles, Calif.

validated for use in horses by determining specificity, accuracy and precision in the measurement of equine cortisol.

Nociceptive thresholds and pupil photographs were obtained at 15 minute intervals during a 30 to 45 minute sampling period and were averaged from all horses at all times. Heart rate, respiratory rate, and rectal temperature were measured continuously. Plasma beta-endorphin and cortisol levels were obtained once immediately prior to the 30 to 45 minute sampling period to avoid exciting the horses. Statistical differences between observations were determined using the Student's paired t-test. Correlations were determined by Pearson's correlation coefficient. Significance was assigned at the $P<0.05$ level.

C. RESULTS

The results revealed a diurnal rhythm in most responses characterized by marked increases or decreases between 0800 and 0900 hours.

1. Nociceptive Thresholds

The STRL and HWRL were both significantly longest at 0900 hours ($p<0.05$) with smaller, secondary peaks at 1500 hours (Figures 5 and 6). These increases were preceded and followed by marked decreases. However, the secondary peaks did not differ significantly from the peaks at 0900 hours nor did they differ significantly from latencies at other times.

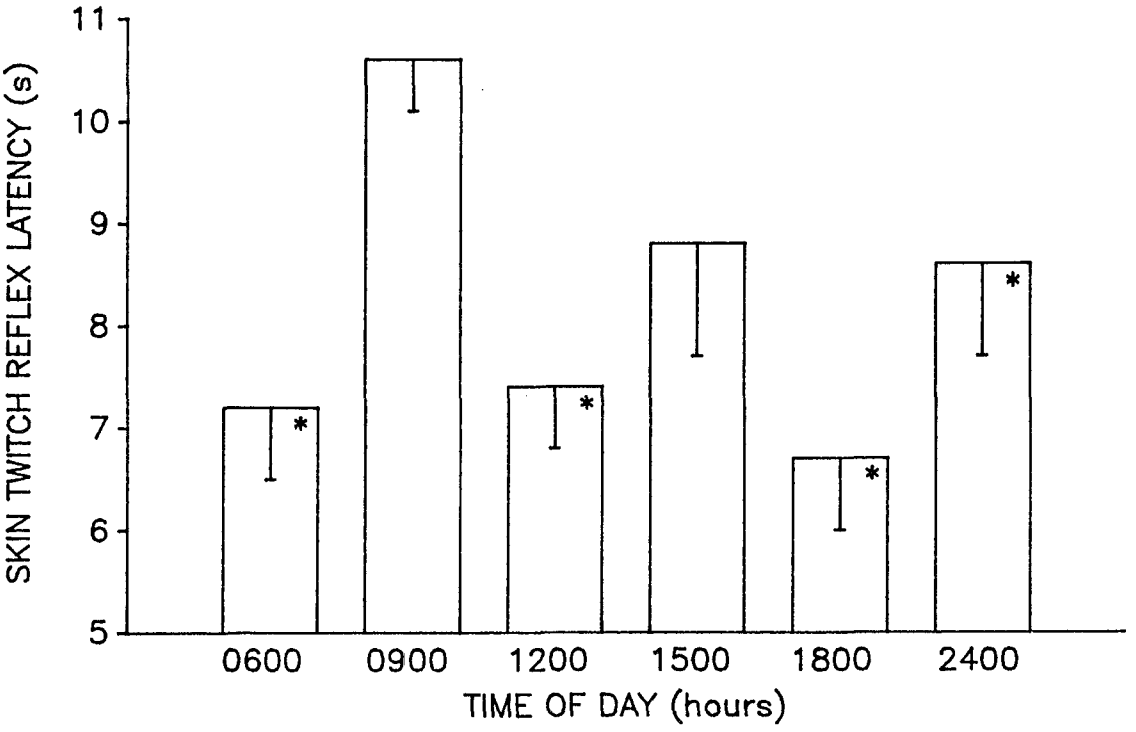

Figure 5. Thermal evoked skin-twitch reflex latencies over 24 hours (Mean±SEM, n=8). (*) indicates statistical difference from the value at 0900 hours ($P<0.05$).

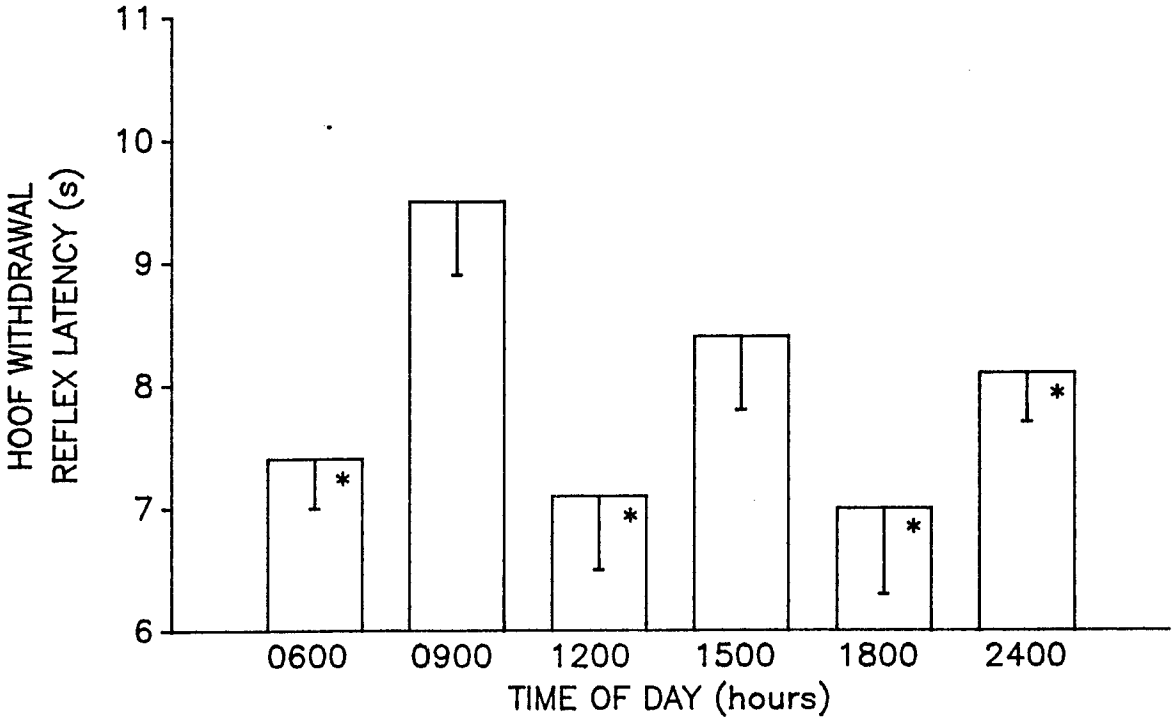

Figure 6. Thermal evoked hoof-withdrawal reflex latencies over 24 hours (Mean±SEM, n=8). (*) indicates statistical difference from the value at 0900 hours ($P<0.05$).

2. Physiologic Responses

Physiologic responses varied greatly. Cardiac rate was elevated at 0900 hours, followed by a significant decrease within three hours (Figure 7). Although the cardiac rate was significantly decreased at 1200 hours, there was another increase at 1800 and 2400 hours which was not statistically different from values at 0900 hours. The respiratory rate was lowest at 0900 hours, preceded by a significantly higher value at 0600 hours (Figure 8). The respiratory rate was also significantly highest at 1800 hours, demonstrating a general trend for higher values at later times of day. Rectal temperature was lowest at 0900 hours and significantly higher at 0600, 1800 and 2400 hours (Figure 9). Here again, there was a general trend for higher values in the evening suggesting that higher body temperatures may result in higher respiratory rates. Vertical pupil diameter was largest at 0900 hours, followed by a marked (6.3 mm) decrease at 1200 hours (Figure 10).

3. Hormonal Levels

Beta-endorphin concentrations were highest at 0900 hours, differing significantly from all other times except 1800 hours (Figure 11). Specificity of the beta-endorphin RIA kit was evaluated by demonstrating parallelism of serial dilutions of equine beta-endorphin and standard solutions. Dilutional parallelism was determined by measuring beta-endorphin levels of serially diluted samples as a determinant of accuracy. Accuracy was also demonstrated by the addition of equine beta-endorphin to equine plasma in concentrations of 5 to 40 pmoles/L (Table 4). The precision of the assay for equine beta-endorphin was comparable to that reported for human beta-endorphin by the manufacturer.

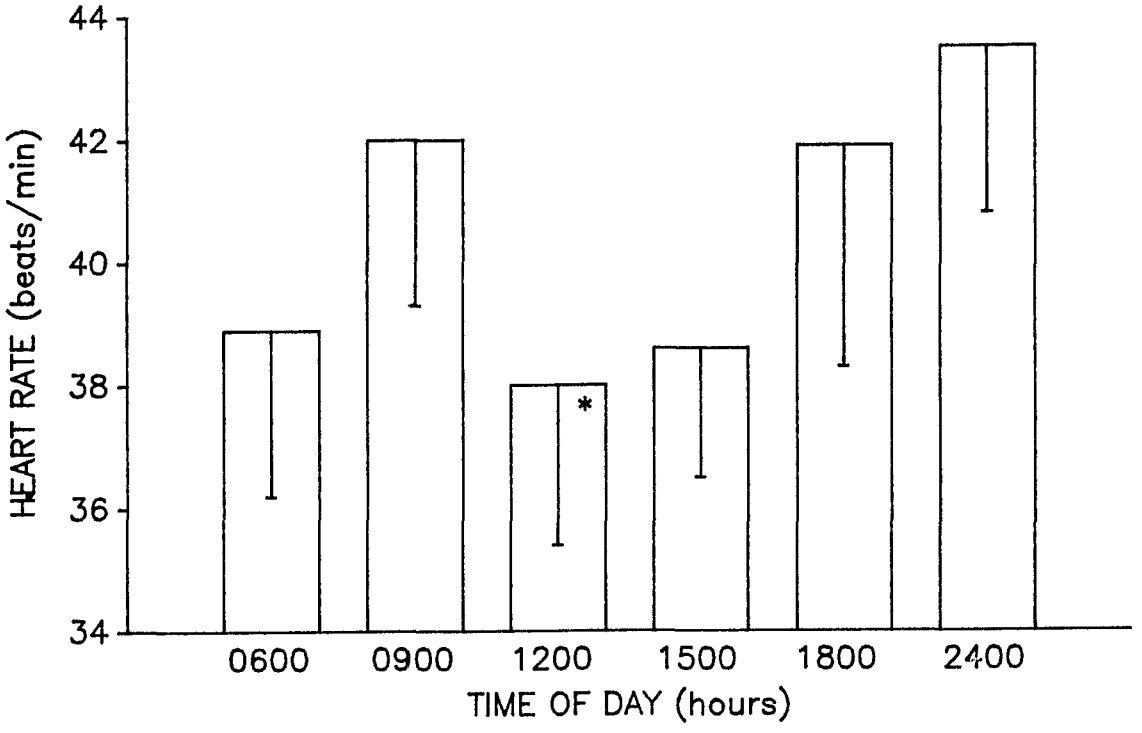

Figure 7. Heart rate over 24 hours (Mean±SEM, n=8). (*) indicates statistical difference from the value at 0900 hours ($P<0.05$).

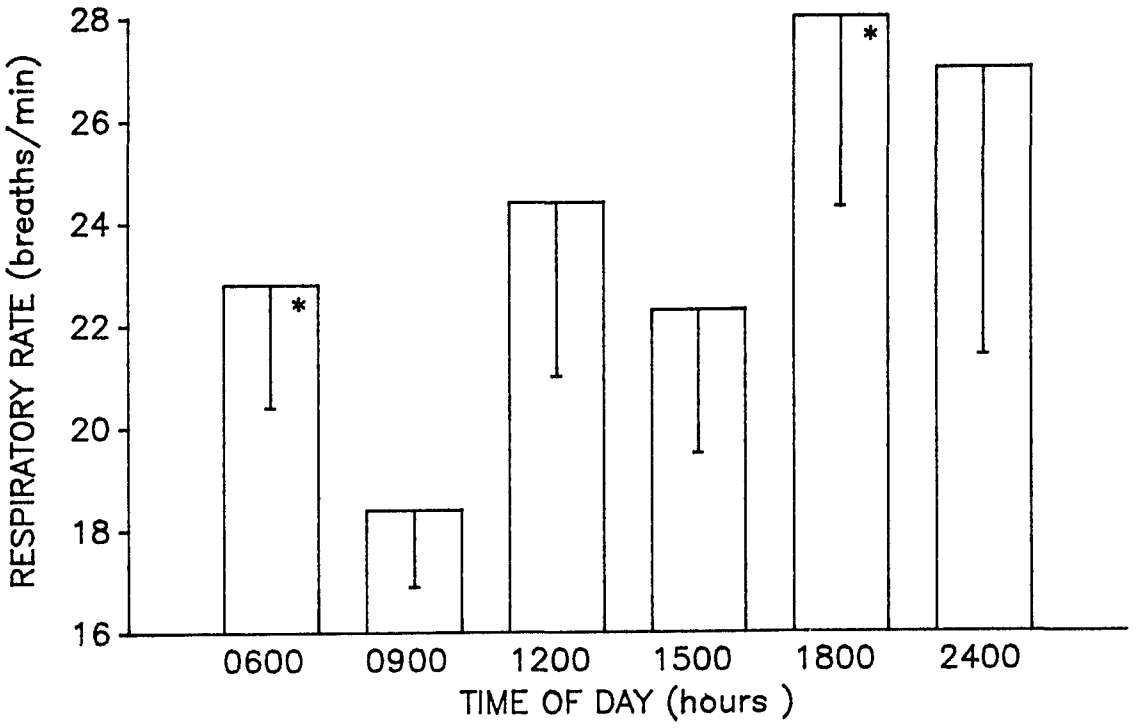

Figure 8. Respiratory rate over 24 hours (Mean±SEM, n=8). (*) indicates statistical difference from the value at 0900 hours ($P<0.05$).

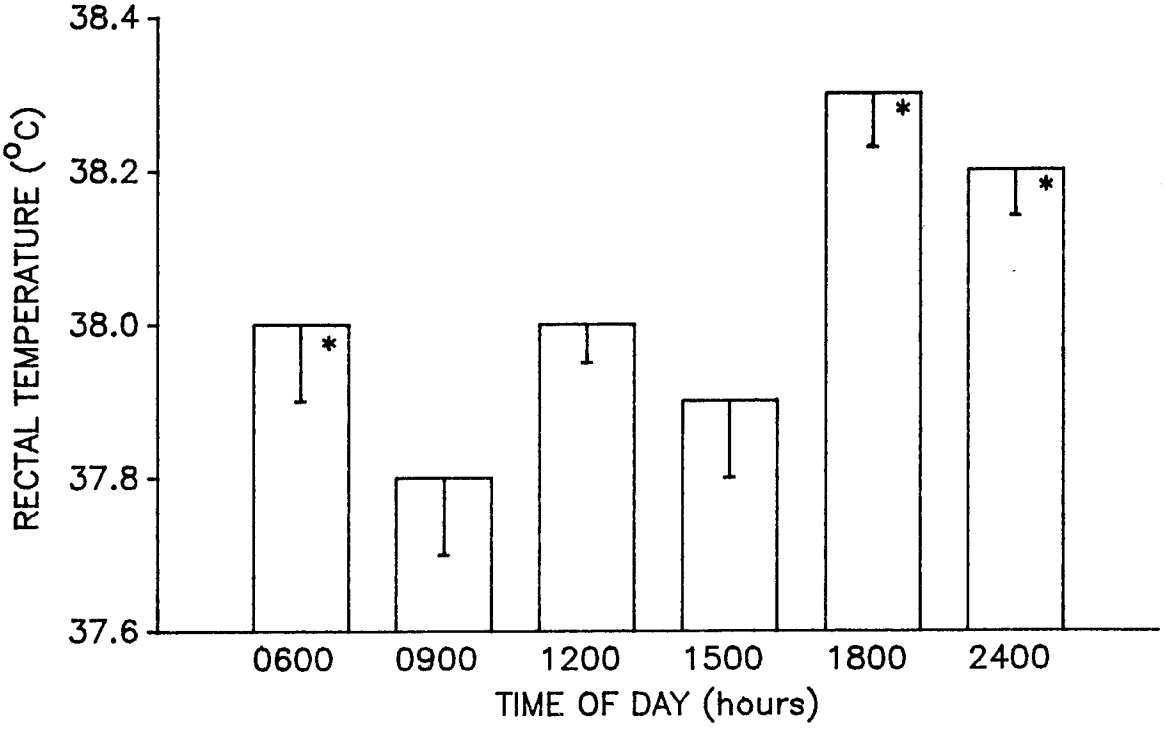

Figure 9. Rectal temperature over 24 hours (Mean±SEM, n=8). (*) indicates statistical difference from the value at 0900 hours ($P<0.05$).

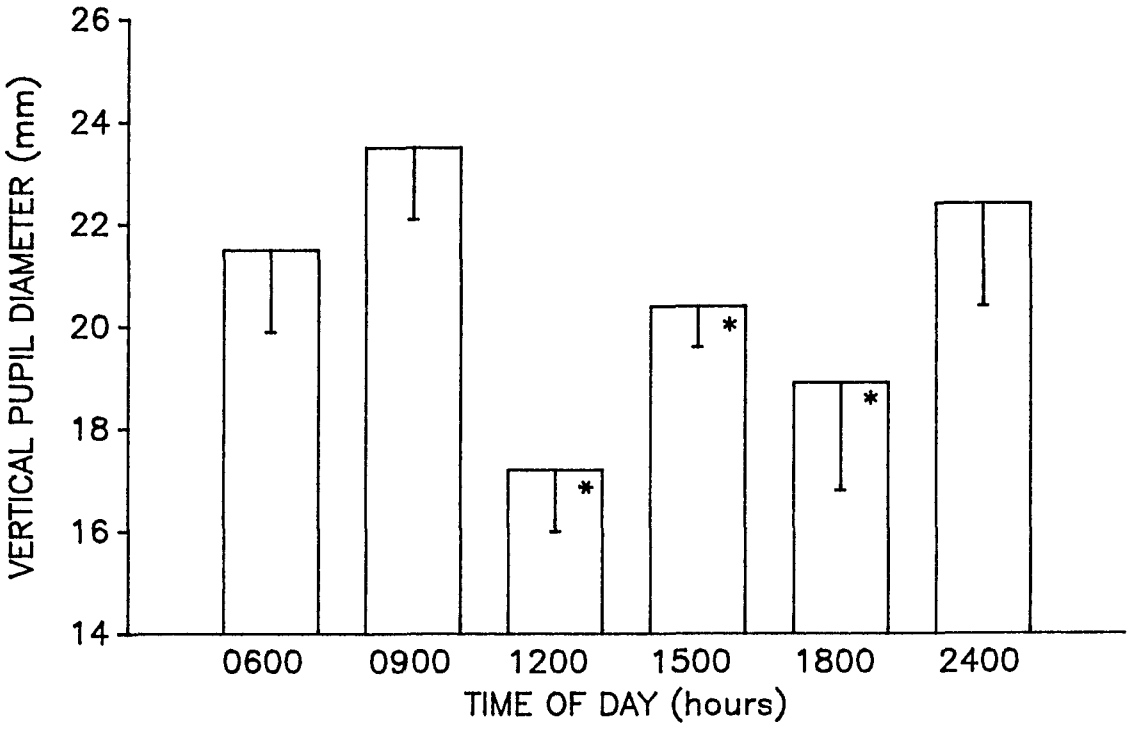

Figure 10. Vertical pupil diameter over 24 hours (Mean±SEM, n=8). (*) indicates statistical difference from the value at 0900 hours ($P<0.05$).

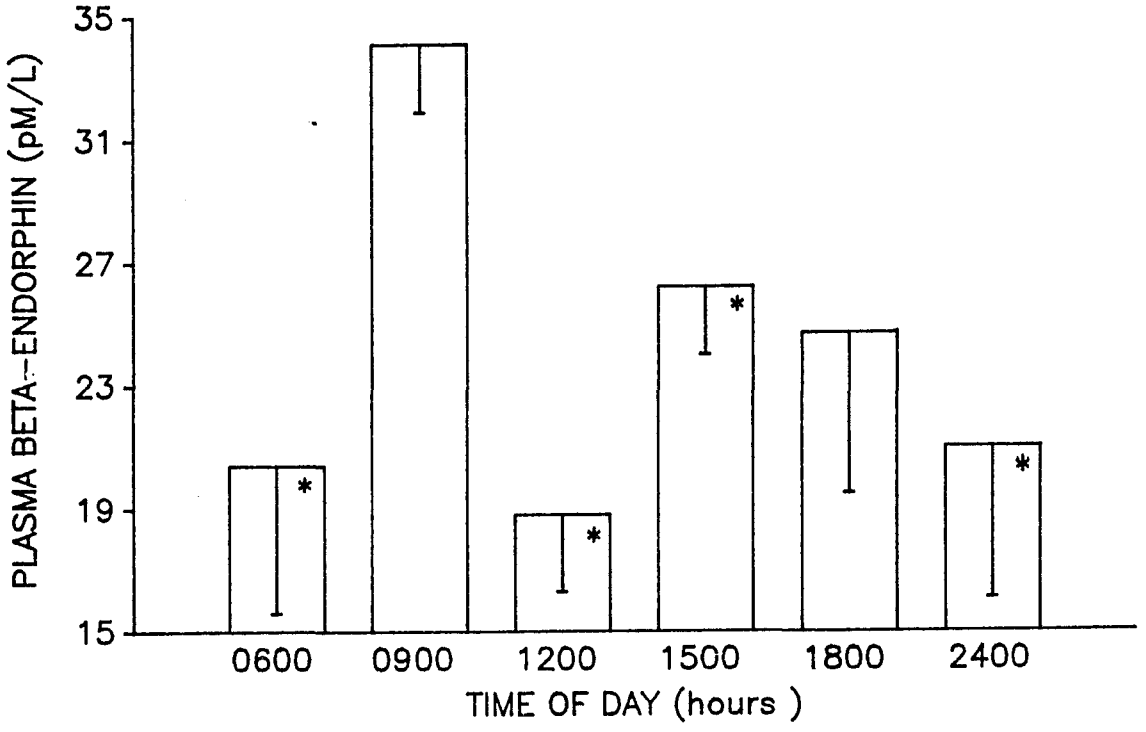

Figure 11. Plasma beta-endorphin concentrations over 24 hours (Mean±SEM), n=8). (*) indicates statistical difference from the value at 0900 hours ($P<0.05$).

TABLE 4. RIA PERFORMANCE DATA

Dilutional Parallelism*

Undiluted	1:2	1:4
40	41	32
20	22	21

Accuracy*

Background	Beta-endorphin Added	Expected Value	Measured Value	Recovery Percent
6.3	5.0	11.33	11.55	102
6.3	10.0	16.33	17.75	109
6.3	20.0	26.33	28.75	109
6.3	40.0	46.33	42.00	91

*Values = pmol/L

The within assay or intra-assay variation yielded a coefficient of variation of 12.2%, which was in the range reported by the manufacturer (6.5-13.7%). The between assay or inter-assay variation yielded a coefficient of variation of 18.8%, which was also comparable to that reported by the manufacturer (18.1%).

Cortisol concentrations were highest at 0800 hours followed by a significant decrease following at 0900 hours (Figure 12). Specificity for equine cortisol was evaluated by demonstrating dilutional parallelism between standard solutions and serial dilutions of endogenous cortisol. Biological specificity was evaluated by demonstrating a two-fold increase in endogenous cortisol following the injection of 100 IU IM of ACTH (6.0 to 14.3 ug/dl). Accuracy was demonstrated by the addition of cortisol to equine plasma in concentrations of 1.05 to 50.0 ug/dl. Linear regression analysis of the recovery curve resulted in a correlation coefficient of 0.9999. The intra-assay precision at three concentrations (1.87, 11.37, 43.50 ug/dl) revealed coefficients of variation of 14.7%, 5.0% and 3.7%, respectively. The interassay coefficients of variation ranged from 4.2% to 16.5%.

4. Correlations

There was a positive correlation between the HWRL and the STRL across all times (Table 5). There was also a strong positive correlation between beta-endorphin concentrations and the STRL. There was a positive correlation between the cortisol concentrations and both the STRL and HWRL. The heart rate and respiratory rate demonstrated a positive correlation across all times. The correlations between beta-endorphin and the STRL and between the STRL and HWRL were stronger than those

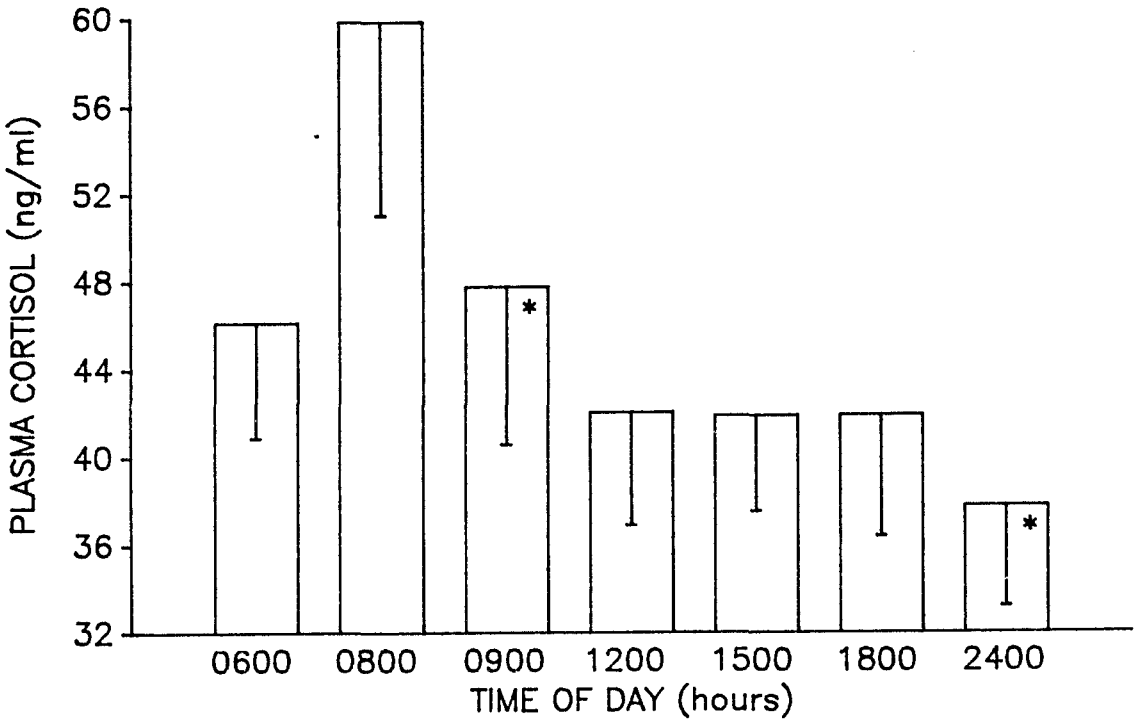

Figure 12. Plasma cortisol concentrations over 24 hours (Mean±SEM, n=8). (*) indicates statistical difference from the value at 0800 hours ($P<0.05$).

TABLE 5. Correlation Coefficients for Diurnal Physiologic and Nociceptive Parameters over 24 Hours

Comparison	r_{obs}	Prob(r>[r_{obs}/H_o: p=0)*
STRL vs. HWRL	0.49488	0.0003
STRL vs. Beta-endorphin	0.51200	0.0002
Cortisol vs. STRL	0.34992	0.0148
Cortisol vs. HWRL	0.30104	0.0376
Heart rate vs. Respiratory rate	0.36702	0.0103

*Probability estimate of acceptance of the null hypothesis.

noted between cortisol and the pain threshold measurements or physiologic parameters.

D. DISCUSSION

The increased skin-twitch and hoof-withdrawal reflex latencies at 0900 hours demonstrated a diurnal rhythm in nociceptive sensitivity. The smaller increases in nociceptive thresholds seen at 1500 and 2400 hours suggest an underlying episodic fluctuation in pain sensitivity throughout the day. The positive correlation between the hoof-withdrawal reflex and the skin-twitch reflex latencies emphasizes the consistent results obtained using two different techniques in the measurement of pain threshold. The significantly increased beta-endorphin concentrations at 0900 hours also demonstrated a diurnal rhythm in plasma beta-endorphin concentrations. The strong positive correlation between the beta-endorphin concentrations and the skin-twitch reflex latencies suggests that diurnal fluctuations in endogenous opioid levels may mediate the diurnal rhythm in nociceptive sensitivity. These results substantiate reports by other investigators which suggest that endogenous opioids may be involved in diurnal fluctuation of nociceptive responses [Wright, 1981; Frederickson, *et al.*, 1977].

It is well known that adrenocorticotropin (ACTH) is released in a diurnal rhythm and beta-endorphin is coreleased with ACTH from the pituitary in response to the hypothalamic peptide, corticotropin-releasing factor [Rivier, *et al.*, 1982]. Adrenocorticotropin is important in controlling the diurnal rhythm of cortisol and therefore has many physiologic effects. Although the cortisol concentrations peaked prior to that of beta-endorphin, cortisol concentrations varied diurnally in the horse in a manner

which approximately paralleled that of beta-endorphin. The difference in the times at which beta-endorphin and cortisol peaked may be resolved by more frequent sampling times of both cortisol and beta-endorphin. Also, the measurement of ACTH rather than cortisol may provide a more sensitive indicator of hormonal fluctuations. However, cortisol concentrations were also positively correlated with both the skin-twitch reflex and the hoof-withdrawal reflex latencies. These results suggest that cortisol may also be involved in the modulation of pain through anti-inflammatory effects at the peripheral level. Corticotropin-releasing factor, which stimulates the release of beta-endorphin and ACTH, may also be involved in pain perception as the administration of CRF produces analgesia in humans and rats [Hargreaves, *et al.*, 1987].

The results obtained from the validation procedures demonstrated accurate, precise measurements of equine beta-endorphin and cortisol using commercially available human RIA kits. Although equine beta-endorphin differs from human beta-endorphin by three amino acid residues [Li, *et al.*, 1981], these residues are apparently not located in the antigenic determinant portion of the molecule. Therefore, the antibody used in this kit recognizes equine as well as human beta-endorphin. Validation of these kits will permit investigators to accurately measure equine beta-endorphin and cortisol with convenient commercially available RIA kits rather than with individually developed assays. Thus, the use of these kits could reduce the variability in results reported from different laboratories and help to clarify the quantitative levels of beta-endorphin release.

The diurnal rhythm of beta-endorphin in the bloodstream may indicate a role for this peptide in other physiologic systems besides that of pain. Beta-endorphin, by acting

on opioid receptors, has many physiologic actions and therefore may control the diurnal rhythm of some physiologic parameters. Morphine is the prototypic mu-opiate receptor agonist and should produce similar responses to those of beta-endorphin which also binds to mu receptors. The changes observed in certain parameters resemble morphine effects in the horse. We observed analgesia, increased heart rate, and increased pupil diameter, all of which have been observed effects of morphine and other mu-agonists in the horse [Muir, *et al.*, 1978; Muir, *et al.*, 1980; Kamerling, *et al.*, 1985a]. Respiratory rate is also increased by morphine and other narcotic agents in the horse [Muir, *et al.*, 1978]. However, this is contrary to our results which showed decreased respiratory rate when analgesia and beta-endorphin levels were greatest. Rectal temperature was also decreased at this time and the decreased body temperature may have influenced respiratory rate. Perhaps the diurnal change of rectal temperature and respiratory rate is not controlled by change in opioid peptide levels. This would explain why the diurnal change in rectal temperature and respiratory rate do not mimic morphine effects in the horse.

The results of this study suggest that beta-endorphin, or other endogenous opioid peptides, may modulate pain threshold as well as other physiologic parameters. Further investigation into the role of opioid receptors in diurnal rhythms needs to be explored. Experiments using naloxone, an opiate receptor antagonist, would help to clarify the role of opioid receptors play in the diurnal rhythm of pain threshold and a number of physiologic parameters. Reversal or inhibition by naloxone of diurnal changes would indicate that there is opioid receptor and opioid peptide involvement. However, the use of naloxone would not necessarily clarify the role of endogenous opioid peptides that are

unable to cross the blood brain barrier (i.e. beta-endorphin) in pain threshold changes.

In conclusion, a diurnal rhythm in both nociceptive thresholds and beta-endorphin levels has been demonstrated in the horse, with peaks at 0900 hours. These results suggest that beta-endorphin may modulate pain as well as other physiologic responses such as heart rate and pupil diameter.

CHAPTER FOUR

EXERCISE INDUCED CHANGES IN BETA-ENDORPHIN LEVELS IN THE HORSE

A. INTRODUCTION

Intense physical exercise has been associated with increases in peripheral blood levels of beta-endorphin in humans [Colt, *et al.*, 1981; Farrell, 1985]. Current studies suggest that beta-endorphin levels increase significantly after moderately high intensity exercise (i.e. 80% VO_{2max}) or maximal exercise intensity (i.e. graded exercise test to exhaustion) [Gambert, *et al.*, 1981; Goldfarb, *et al.*, 1987]. Submaximal exercise intensity (i.e. 60% VO_{2max} or lower) does not consistently alter beta-endorphin levels [Rahkila, *et al.*, 1987; Langenfeld, *et al.*, 1987]. Recent research suggests that exercise training augments beta-endorphin release following high intensity exercise, although not all studies support this theory [Carr, *et al.*, 1981; Mougin, *et al.*, 1987; Howlett, *et al.*, 1984]. The question arises as to whether the absolute exercise intensity alone which is attainable only by trained athletes is responsible for the increased levels of beta-endorphin noted in the majority of these studies. Considerable variation exists in the methods used to detect beta-endorphin or beta-endorphin-like immunoreactivity in plasma. The different methods used to measure beta-endorphin concentrations have led to reports of beta-endorphin concentrations varying from 10 pg/ml to 1.6 ng/ml [Farrell, 1985]. Therefore, while many of these studies may correctly indicate a change in beta-endorphin concentrations, the exact magnitude of change may not be accurate [Sforzo, 1989].

Beta-endorphin concentrations following exercise in the horse have been examined by few investigators. Evans *et al.* [1985] reported increased plasma beta-endorphin concentrations in the horse following a strenuous gallop with levels returning to baseline within a half hour. Li and Chen [1987] demonstrated incremental increases in beta-endorphin concentrations following a trot, slow gallop and fast gallop in Thoroughbred horses. Although Li and Chen [1987] reported approximate speed, no other means of evaluating exercise intensity were reported by these investigators. Also, both groups of investigators used radioimmunoassay techniques to measure equine beta-endorphin without reporting adequate validation of these methods in the horse. Therefore, although increases in beta-endorphin concentrations occur following exercise in the horse, the intensity of exercise which stimulates a response and the exact magnitude of the response have yet to be addressed. Also, the role of training in the release of beta-endorphin has not been examined in the horse. Therefore this study was undertaken in order to determine the role of exercise intensity and physical conditioning in the release of beta-endorphin in the horse.

B. MATERIALS AND METHODS

Horses

Ten healthy Thoroughbred horses, five males and five females, with a mean age of 4.5$\pm$0.5 (Mean$\pm$S.D.) years were used in this study. All horses had received previous exercise training but had not been raced or trained for nine months prior to this study. The horses were clinically normal as determined by a complete blood count, serum

biochemistry profile and physical examination.

Exercise testing and conditioning regimen

Horses were subjected to 5 exercise tests of maximal intensity under varying conditions. The first two exercise tests were performed on horses in an unconditioned or unfit state which was followed by nine weeks of a conventional race conditioning program. Following the conditioning period, three more exercise tests were performed. Exercise tests were begun at approximately 0730 hours and completed by 1000 hours. The exercise tests were spaced at 10 day intervals to allow for recuperation. The experimental timetable and conditioning program is shown in Table 6.

Horses were fed their evening rations on the day before each exercise test and food was then withheld prior to testing. Blood samples were collected from the jugular vein at rest in their stalls before testing. The animals were then saddled, taken to the track, and ridden by the same experienced jockey. Each horse was then trotted for 1200 meters and cantered for 800 meters in a clockwise direction (warm-up). The animals were then stopped, turned in the opposite direction and asked for a maximal effort run for 400 or 1000 meters depending on the test. Exercise intensity and the degree of exertional stress were evaluated by heart rate, respiratory rate, lactate concentrations and pain threshold measurements. Naloxone pretreatment was used to evaluate opioid involvement in the horses' response to the exertional stress. Naloxone hydrochloride[1] was dissolved in a 0.9% sterile sodium chloride solution and injected intravenously immediately prior to exercise. In the 1000 meter exercise tests, either naloxone or an equal volume of

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[1]Naloxone hydrochloride, Sigma Chemical Company, St. Louis, MO.

TABLE 6. Experimental Timetable

Week	
1	Alternate daily 400m trot or 80m swim
2	Preconditioning exercise test - 400m
3	Preconditioning exercise test - 400m Naloxone pretreatment (0.75 mg/kg IV)
4-13	Horses conditioned 6 days/week: 1600m completed over 4-5 mins/day (5.3-6.7 m/sec) for 4 days/week, adding 800m each week until 4000m/day was performed. Speed increased over 9 weeks until 8.9m/sec was reached. Horses swum 2 days/week, increasing to 24 laps in 20 mins of an 11m pool by week 13.
14	Postconditioning exercise test - 400m
15 and 16	Postconditioning exercise tests - 1000m Naloxone (0.75 mg/kg IV) or saline (0.9%, IV) pretreatment

saline was injected prior to the exercise test in a double-blind crossover design. Blood samples for beta-endorphin, cortisol and lactate were taken before, immediately after and 30 minutes after each exercise test. Measurements of pain sensitivity were obtained before and at 2, 10, 20 and 30 minutes after exercise using the method of Kamerling, *et al.* [1985] (Refer to Chapter 3 for detailed description of the procedure). Heart rate was measured using an on board heart rate monitor[2]. Respiratory rate was determined from polygraph recordings using impedance pneumography[3]. Heart rate and respiratory rate measurements were taken for 10 minutes prior to the exercise tests and continuously for thirty minutes following the exercise tests. Rectal temperature was recorded from a deep rectal probe and a digital thermometer before and immediately after each exercise test. Ambient temperature was recorded at the time of each exercise test using a digital thermometer.

Sample Analysis

Blood samples for plasma beta-endorphin and cortisol levels were collected into 7 ml evacuated siliconized glass tubes containing EDTA as an anticoagulant. Blood samples for lactate concentrations were collected in 4 ml evacuated glass tubes containing sodium fluoride as a preservative. The samples were immediately centrifuged and the plasma or serum removed and frozen at -20°C until the assays could be performed. Plasma beta-endorphin levels were measured using a commercially available

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[2]Equistat Model HR-7A, EQB Inc., Unionville, Penn.

[3]Grass polygraph Model 7, Low-level DC pre-amplifier Model 7P1FG, Grass Instruments Co., Quincy, Mass.

radioimmunoassay (RIA) kit. Cortisol concentrations were also determined using a commercially available RIA kit (For detailed procedures and validation of beta-endorphin and cortisol RIA kits, refer to Chapter 3). Lactate concentrations were determined using a commercially available kit[4]. The lactate procedure was based on the measurement of the generation of the reduced form of nicotinamide adenine dinucleotide (NADH) spectrophotometrically at 340 nm. In the presence of excess nicotinamide adenine dinucleotide (NAD) and the enzyme lactate dehydrogenase, lactate was converted to pyruvate along with the generation of NADH, which caused increased absorbance at 340 nm. A protein precipitant, 8% perchloric acid, was used initially on the sample and the supernatant was then used directly without further treatment. Lactates were measured on a narrow-bandwidth spectrophotometer[5].

Statistical Analyses

Data were analyzed by multiple analysis of variance for repeated measures followed by Duncan's multiple range test and Pearson's correlation coefficients. Student's paired t-test was also performed where applicable. Significance was assigned at the $P<0.05$ level.

C. RESULTS

Intense physical exercise resulted in significantly increased concentrations of beta-endorphin after all exercise tests when compared to levels obtained at rest (Figure 13).

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[4]Sigma Diagnostics, Sigma Chemical Co., St. Louis, Mo.

[5]Gilford Spectrophotometer, Model 250, Gilford Instrument Laboratories, Oberlin, Ohio.

Conditioned horses subjected to a 400m exercise test had significantly lower beta-endorphin levels at 30 minutes post exercise than did unfit horses (Figure 13, comparison a) ($P<0.05$). These results indicate a more rapid return to baseline levels in the conditioned group. An increase in distance to 1000m resulted in increased beta-endorphin concentrations compared to levels following a 400m exercise test (Figure 13, comparison b) ($P<0.01$). These results suggest that the increased distance resulted in an increase in exercise intensity and therefore suggest a correlation between exercise intensity and beta-endorphin concentrations following exercise. Naloxone pretreatment resulted in increased beta-endorphin concentrations in unfit horses 30 minutes following exercise as compared to unfit horses without naloxone pretreatment (Figure 13, comparison c) ($P<0.05$). This phenomenon was also noted in fit horses 30 minutes following a 1000m exercise test (saline vs. naloxone, $P<0.05$).

Cortisol concentrations were also elevated following exercise although conditioning did not significantly alter the response (Figure 14). Cortisol concentrations were significantly increased over baseline values after all times except immediately post exercise following a 400m exercise test in the unconditioned horses. The increase in distance from the 400m to 1000m exercise test resulted in higher levels 30 minutes post exercise ($P<0.05$) but not immediately post exercise (Figure 14, comparison a). These results again suggest a correlation between exercise intensity and hormonal concentrations. Significantly higher cortisol levels ($P<0.01$) were noted immediately following exercise in unconditioned, naloxone pretreated horses suggesting that naloxone pretreatment resulted in a faster rise in cortisol concentrations following exercise in unconditioned

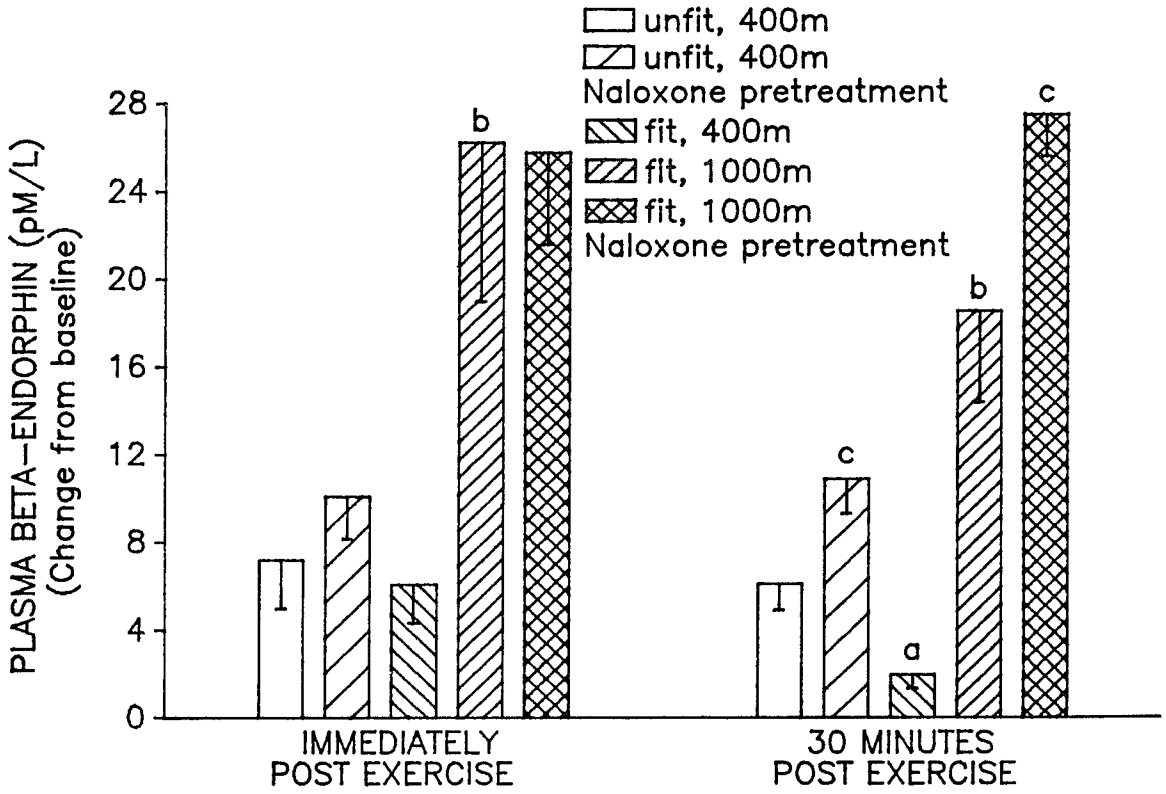

Figure 13. Change in plasma beta-endorphin concentrations from baseline following exercise. Letters indicate statistical differences ($P<0.05$): (a) fit vs. unfit, 400m, 30 minutes post exercise, (b) fit, 400m vs. 1000m, (c) naloxone vs. saline.

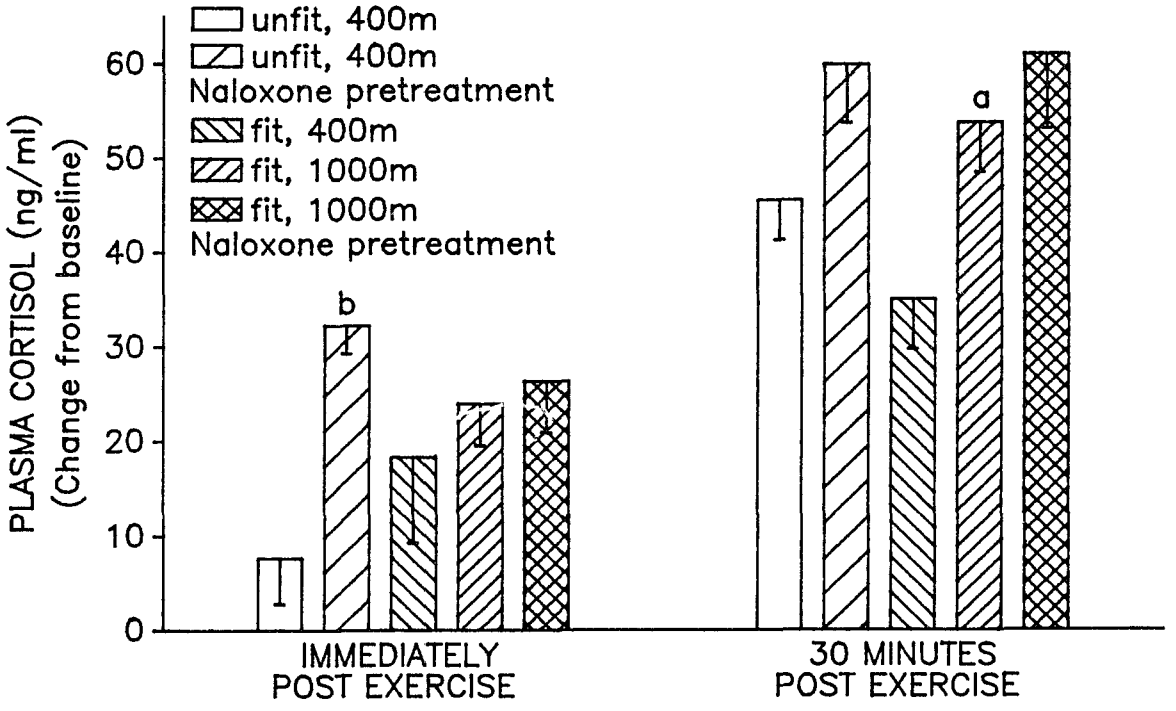

Figure 14. Change in plasma cortisol concentrations from baseline following exercise. Letters indicate statistical difference ($P<0.05$): (a) fit, 400m vs. 1000m, (b) unfit, 400m, no pretreatment vs. naloxone pretreatment.

horses (Figure 14, comparison b). There was no difference in cortisol concentrations following naloxone pretreatment from saline pretreatment in conditioned horses.

The assessment of exercise intensity and the degree of exertional stress were analyzed by measuring pain threshold, heart rate, respiratory rate and blood lactate concentrations. Intense exercise resulted in significantly increased pain thresholds in the unconditioned horse at 2 minutes post exercise ($P<0.01$) (Figure 15). Naloxone pretreatment prior to the 400m exercise test in the unconditioned horses resulted in a significant decrease in pain threshold from baseline values at 10, 20 and 30 minutes post exercise. These results suggest that naloxone pretreatment in unconditioned horses results in hyperalgesia following intense exercise. The 400m exercise test in conditioned horses did not significantly elevate pain threshold although the increased distance of the 1000m exercise test did significantly increase pain threshold at 2 minutes post exercise ($P<0.05$). The increased pain threshold noted after 1000m in the fit horse was not as great as that seen after 400m in the unconditioned horse. Naloxone pretreatment reversed the analgesia noted following the 1000m exercise test in conditioned horses but did not result in hyperalgesia. Interestingly, the elevations in pain threshold occurred within two minutes post exercise and rapidly returned to baseline while the hyperalgesic effect of naloxone was not seen until 10 minutes post exercise and persisted for 30 minutes.

Heart rate was significantly increased following all exercise tests ($P<0.001$), with the highest peak values seen immediately following the 400m exercise test in the unconditioned horses (Figure 16, comparison a, Table 7). Conditioning significantly decreased the peak heart rate following the 400m exercise test ($P<0.01$) and decreased the

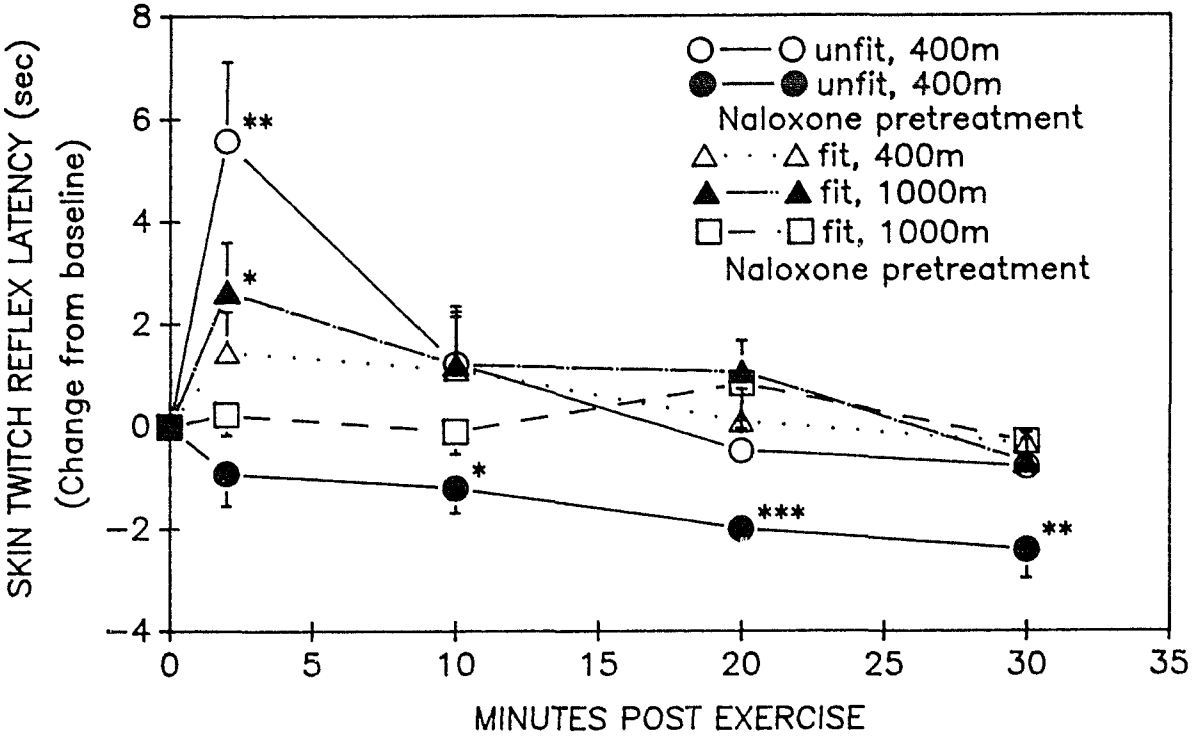

Figure 15. Change in skin-twitch reflex latency from baseline over a 30 minute period following exercise. (*) indicates statistical difference from baseline: * $P<0.05$, ** $P<0.01$, ***$P<0.001$.

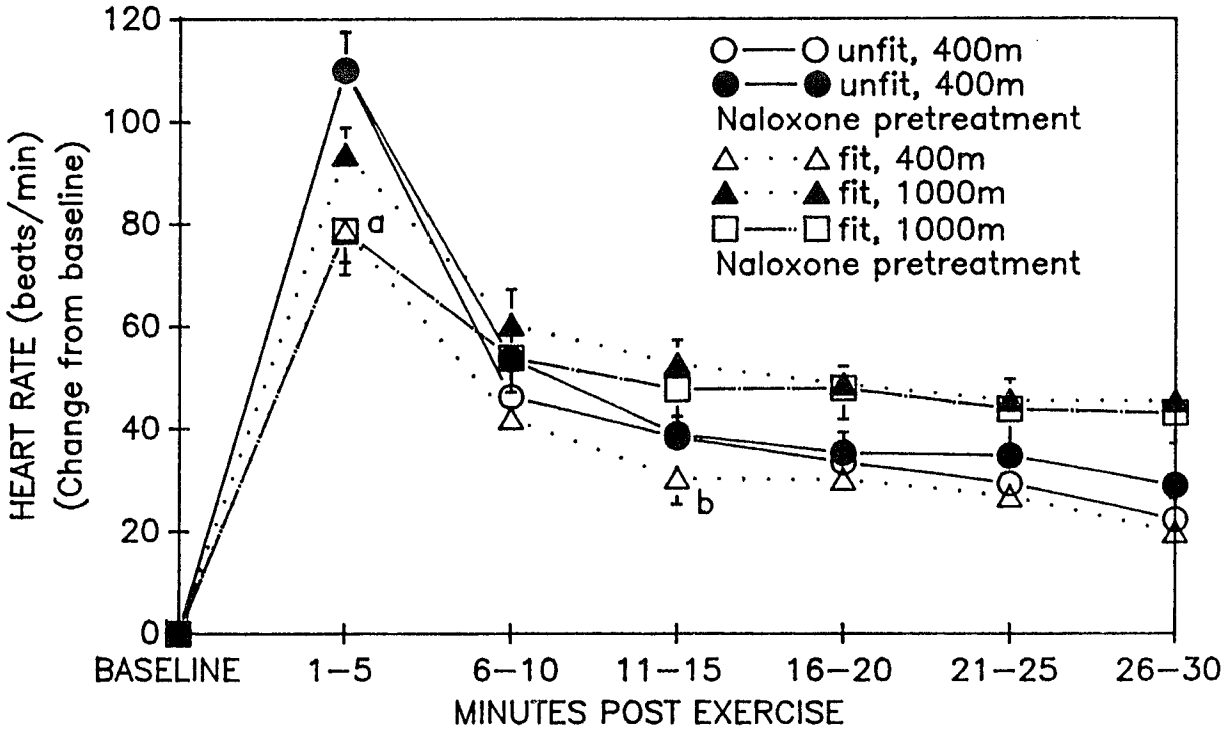

Figure 16. Change in heart rate from baseline following exercise, averaged over 5 minute intervals. Letters indicate statistical difference ($P<0.05$): (a) unfit vs. fit, 400m, 1-5 min., (b) unfit vs. fit, 400m, 11-15 min.

TABLE 7. Peak Heart Rates

Exercise Test	**Peak Heart Rates** (Mean±SEM) (Beats/min)
Unfit, 400m	245.7±11.5
Unfit, 400m, Naloxone pretreatment	228.9±8.20
Fit, 400m	213.7±11.1
Fit, 1000m	182.6±5.80
Fit, 1000m, Naloxone pretreatment	162.1±2.50

time for the heart rate to return to baseline or recovery time ($P<0.05$ at the 11-15 minute interval). Heart rate was also significantly elevated over all times following the 1000m exercise tests as compared to the 400m exercise tests in the conditioned horses ($P<0.01$). Naloxone pretreatment did not alter heart rate response to exercise in either the unconditioned or conditioned horse. An average peak heart rate of 201 ± 8 beats/min was noted following all exercise tests.

Respiratory rate was also significantly increased following all exercise tests ($P<0.001$) (Figure 17) while respiratory rates following the 1000 m exercise tests were significantly higher than those following the 400 m exercise tests in unfit horses ($P<0.05$). Respiratory rates following the 400m exercise test in fit horses were also significantly higher when compared to the 400m test in unfit horses ($P<0.05$). Naloxone pretreatment had no significant effect on respiratory rate. Respiratory rates following the 1000m exercise test in naloxone pretreated horses appeared higher than saline pretreated horses but were not statistically significant. Lactate concentrations were significantly increased over baseline values ($P<0.001$) after all exercise tests, with post exercise values always exceeding 4 mmol/L or anaerobic threshold (Figure 18). There was no statistical difference between lactate concentrations following a 400m exercise test in unconditioned horses when compared to conditioned horses. The increased distance associated with the 1000m exercise test resulted in significantly higher concentrations than seen both immediately and thirty minutes following the 400m test in conditioned horses ($P<0.001$) (Figure 18, comparison a). Naloxone pretreatment did not alter the lactate response to exercise in the unconditioned horse following the 400m exercise test but did decrease the

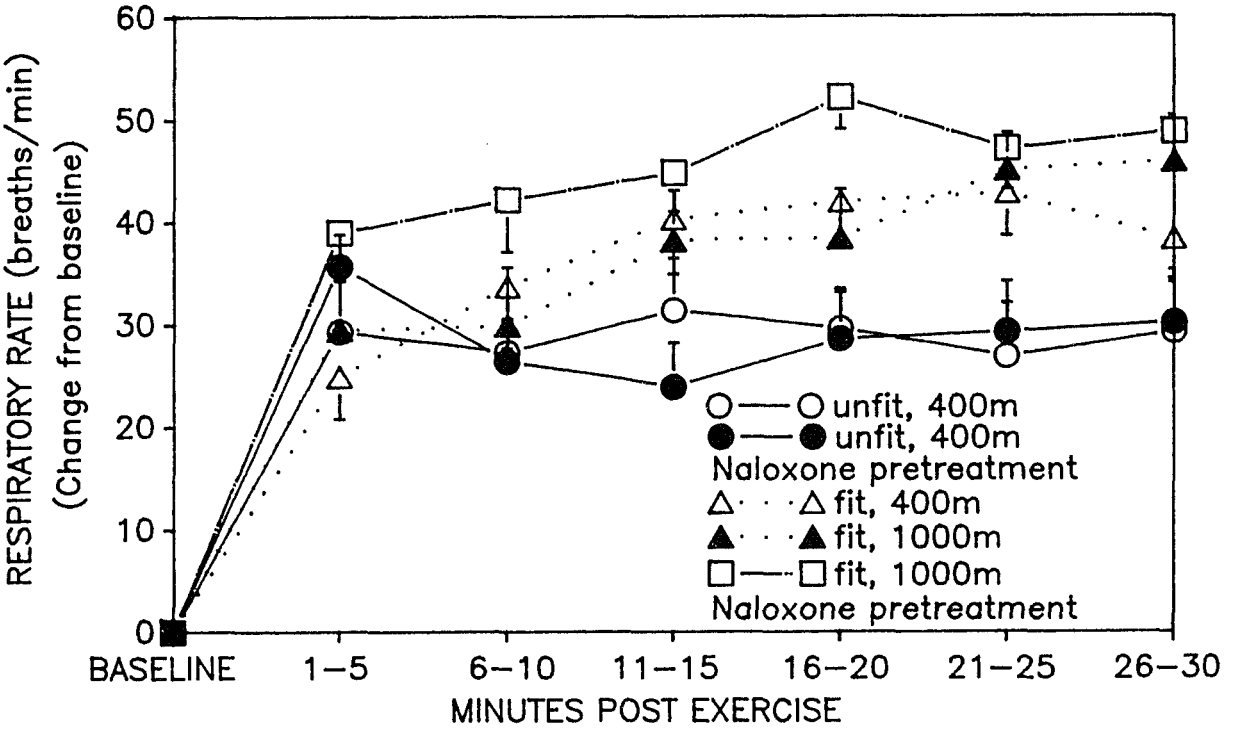

Figure 17. Change in respiratory rate from baseline following exercise, averaged over 5 minute intervals.

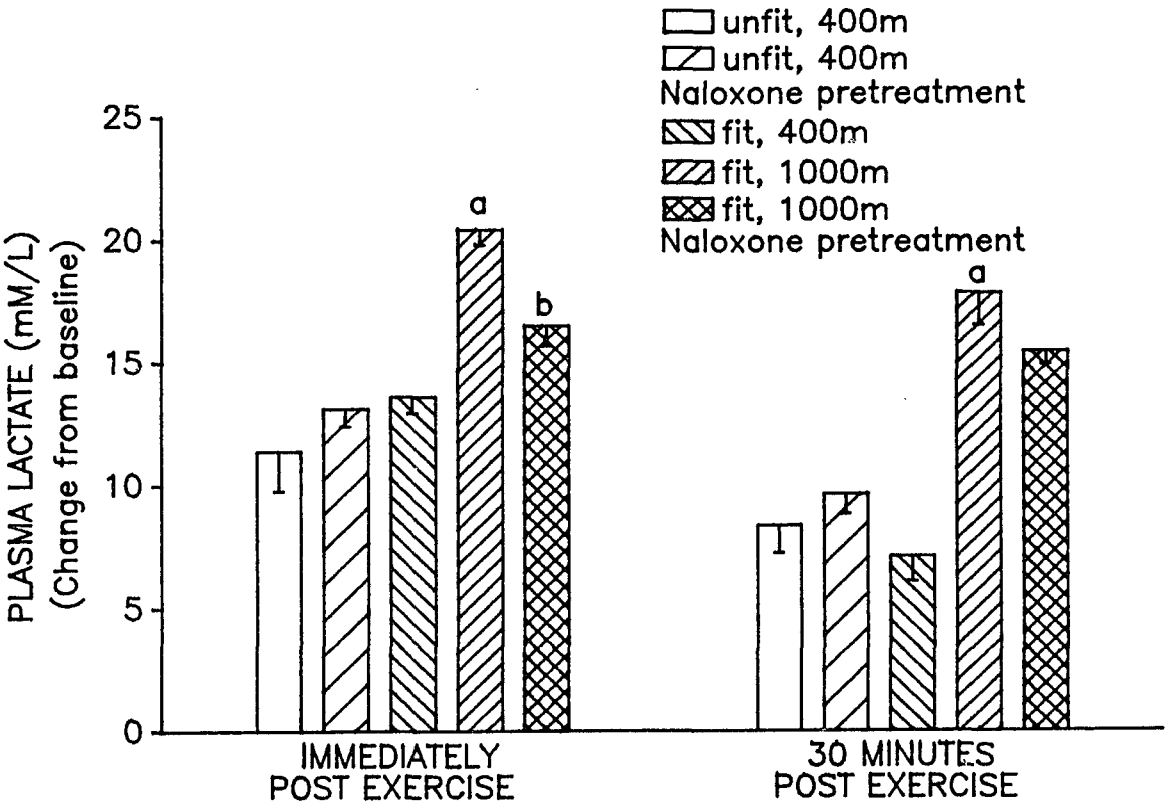

Figure 18. Increase in lactate concentrations from baseline following exercise. Letters indicate statistical difference ($P<0.05$): (a) fit, 400m vs. 1000m, (b) fit, 1000m, naloxone vs. saline.

amount of lactate produced immediately following the 1000m exercise test in the conditioned horse ($P<0.01$) (Figure 18, comparison b).

Rectal temperature increased significantly from baseline values following all exercise tests except the 400m exercise test in conditioned or fit horses ($P<0.01$) (Figure 19). Rectal temperatures following the 1000m exercise test were significantly higher than those noted after the 400m exercise test in fit horses ($P<0.02$). Lower values following naloxone pretreatment were noted but were not statistically significant. Ambient temperature was significantly higher during the exercise tests following the conditioning or training period from the ambient temperature during the tests run prior to the nine week training period ($P<0.001$) (Figure 20).

In unconditioned horses pretreated with naloxone following a 400m exercise test, there was an inverse correlation between the skin twitch reflex latency and beta-endorphin concentrations across all times (Table 8). There was a strong positive correlation between beta-endorphin and lactate concentrations following all exercise tests. There was also a strong positive correlation between lactate concentrations and heart rate following all exercise tests. Lactate concentrations also showed a strong positive correlation with respiratory rates following all exercise tests. Beta-endorphin and cortisol concentrations were positively correlated following exercise tests with naloxone pretreatment.

D. DISCUSSION

Data from human research suggest that moderately high and high intensity exercise stimulate the release of beta-endorphin into the circulation [Sforzo, 1989]. Exercise

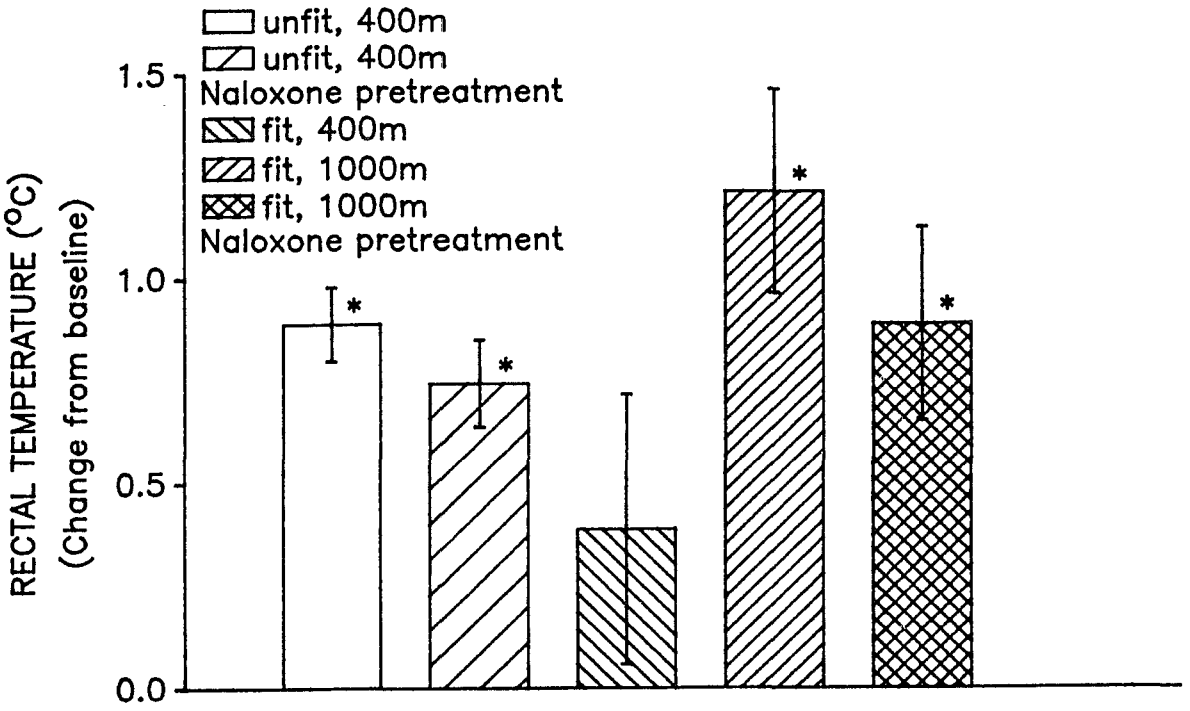

Figure 19. Increased rectal temperature from baseline following exercise. (*) indicates statistically different from baseline ($P<0.01$).

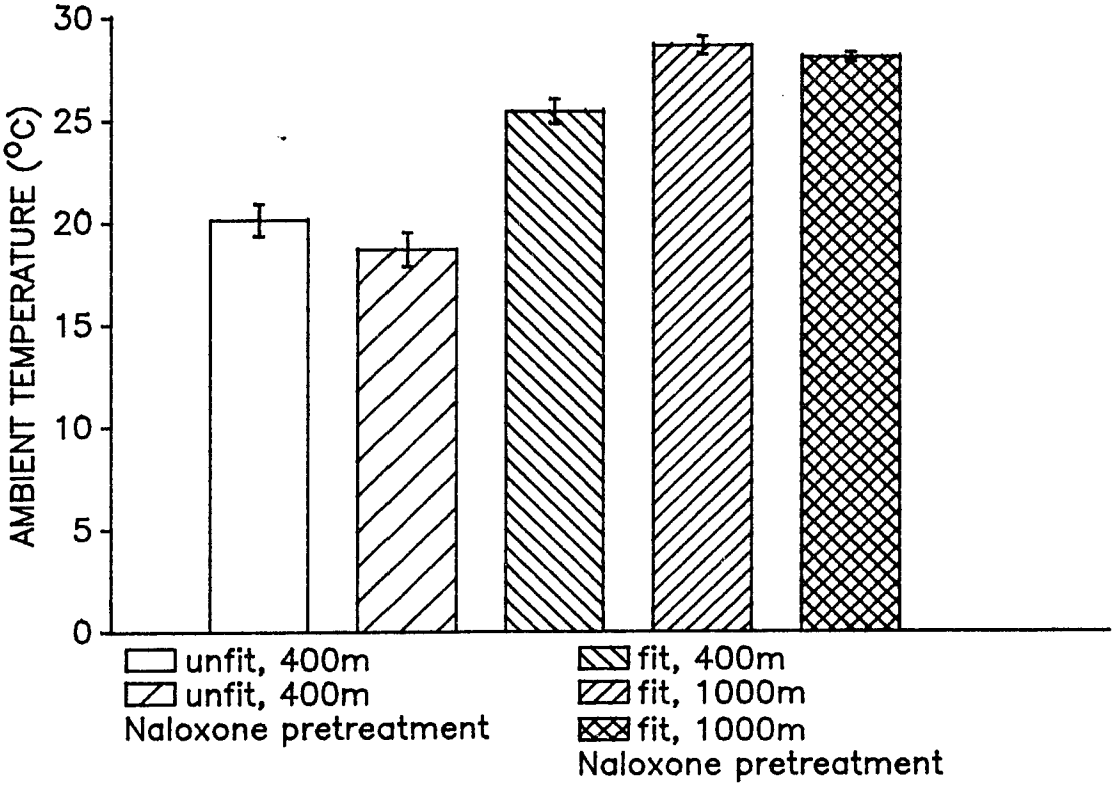

Figure 20. Ambient temperature at the time of each exercise test. (*) indicates statistical difference from exercise tests with unfit horses ($P<0.001$).

TABLE 8. Pearson's Correlation Coefficients for Exercise Test Parameters

Comparison	r_{obs}	Prob(r>[r_{obs}]/H_o: p=0)*
Beta-endorphin vs. Skin twitch Reflex Latency		
Unfit, 400m, Naloxone pretreatment	-0.60080	0.0009
Beta-endorphin vs. Lactate		
Unfit, 400m	0.44542	0.0226
Unfit, 400m, Naloxone pretreatment	0.69779	0.0001
Fit, 400m	0.56732	0.0020
Fit, 1000m	0.68337	0.0001
Fit, 1000m, Naloxone pretreatment	0.86473	0.0001
Beta-endorphin vs. Cortisol		
Unfit, 400m, Naloxone pretreatment	0.72269	0.0001
Fit, 1000m, Naloxone pretreatment	0.66854	0.0001
Lactate vs. Heart Rate		
Unfit, 400m	0.55207	0.0035
Unfit, 400m, Naloxone pretreatment	0.73546	0.0001
Fit, 400m	0.78690	0.0001
Fit, 1000m	0.73986	0.0001
Fit, 1000m, Naloxone pretreatment	0.77263	0.0001
Lactate vs. Respiratory Rate		
Unfit, 400m	0.65090	0.0003
Unfit, 400m, Naloxone pretreatment	0.80561	0.0001
Fit, 400m	0.46958	0.0135
Fit, 1000m	0.74852	0.0001
Fit, 1000m, Naloxone pretreatment	0.81020	0.0001

*Probability estimate of acceptance of the null hypothesis.

intensity was defined in this study by changes in heart rate, respiratory rate, and lactate levels following exercise as an approximation of VO_{2max}. Based on peak heart rates and lactate concentrations, it can be concluded that the horses reached near maximal or maximal exercise intensity following all exercise tests. Peak heart rates averaged 201 beats/min, while lactate concentrations exceeded 4 mmol/L or anaerobic threshold. These conclusions are substantiated by studies which have used heart rates of 200 beats/min in combination with lactate concentrations exceeding 4 mmol/L to define maximal aerobic work power in the horse [Persson, 1983]. Increased peak heart rates, respiratory rates, and lactate concentrations following the 1000 m exercise test when compared to the 400 m exercise test in conditioned or unconditioned horses suggested that the increase in exercise duration resulted in a corresponding increase in exercise intensity. Lactate concentrations were positively correlated with heart rate and respiratory rate in all exercise tests. The correlation of lactate concentrations with heart rate and respiratory rate suggests that increased exercise intensity results in increased lactate concentrations, heart rate and respiratory rate and supports the conclusion that an increase in exercise duration results in increased exercise intensity.

The degree of exertional stress experienced by individuals, following exercise of equal intensity, may vary with each individual. The level of fitness, state of health, and a number of psychological factors are capable of influencing the degree of exertional stress [Cummings and Wheeler, 1987]. Exertional stress in humans is commonly defined by mood alteration, perceived work effort and, in some cases, pain threshold measurements [Janal, *et al.*, 1984; Scott and Gijsbers, 1981; Kemppainen, *et al.*, 1985].

These measurements are usually determined by questionnaires and therefore represent subjective assessments of exertional stress. In this study however, the degree of exertional stress was analyzed by heart rate, pain threshold measurements, hormonal levels and other relevant physiologic parameters, therefore providing more objective means of assessing exertional or exercise stress levels. Peak heart rates were the highest following the 400 m exercise test in the unconditioned horses. Conditioning resulted in a significant decrease in peak heart rates and a faster return to baseline although work load remained the same. Increased exercise duration, which suggested a corresponding increase in exercise intensity (1000 m exercise test), resulted in an increase in peak heart rate from that seen in conditioned horses following the 400 m exercise test. However, the peak heart rates following the 1000 m exercise tests in fit horses were lower that those following the 400 m test in unfit horses. These results suggest that the unconditioned horses experienced a greater degree of exertional stress than the conditioned horses, even when the conditioned horses were subjected to an exercise test requiring increased exercise intensity or work load.

The increased pain threshold following the 400 m exercise test in the unconditioned horses again suggests that the unfit horses experienced a greater degree of exertional stress. The increase in pain threshold or analgesia noted after the 400 m exercise test in unfit horses was not seen in the conditioned horses following the 400 m exercise test. However, increasing exercise duration and/or intensity to 1000 m again elevated pain threshold, although it was not as great as that seen in the unfit horse. These results confirm the conclusions drawn based on the heart rate measurements, that the unfit

horse may experience a greater degree of exertional stress than does the conditioned or fit horse, even when the fit horse is subjected to an exercise test of greater exercise intensity.

Rectal temperature was not significantly increased following the 400 m exercise test in the fit horses, although it was elevated following all other exercise tests. This again supports the theory that the conditioned horses experienced less exertional stress than the unconditioned horses. Respiratory rate analysis did not support this conclusion, as the respiratory rates were higher in the fit horses than the unfit horses. This may be due to the increased ambient temperature during the time of the post-conditioning exercise tests.

Beta-endorphin concentrations increased significantly following all exercise tests. Beta-endorphin levels were lower in all 400 m exercise tests when compared to the 1000 m exercise tests. The higher levels seen following the 1000 m exercise tests suggest that there is a correlation between exercise intensity and beta-endorphin release. However, actual oxygen consumption measurements, along with more rigorously standardized test conditions (i.e., graded treadmill exercise to exhaustion), would be necessary to clearly define a correlation between exercise duration, exercise intensity and beta-endorphin levels.

Beta-endorphin concentrations returned to baseline faster following a 400 m exercise test in fit horses when compared to unfit horses, suggesting a correlation also exists between beta-endorphin concentrations and the amount of exertional stress experienced by the horse. The greatly increased beta-endorphin concentrations noted

following the 1000 m exercise test, when compared to the 400m exercise test, however, suggested that beta-endorphin release is linked more with the degree of exercise intensity than with the amount of exertional stress. If beta-endorphin release was linked primarily with the amount of exertional stress experienced by the horse, the highest beta-endorphin concentrations would have occurred following the 400 m exercise test in unfit horses, which was not the case.

Conditioning did not increase the release of beta-endorphin in response to the 400 m exercise test. However, the increase in exercise intensity and/or duration (1000 m exercise test) did result in a substantial elevation in beta-endorphin concentrations when compared to those following the 400 m exercise tests. Most studies in humans which evaluated the effects of training on beta-endorphin release increased the level of exercise intensity in the trained athletes when compared to exercise tests performed in sedentary individuals. The studies which did not increase exercise intensity did not demonstrate increase releasability, with one exception [Sforzo, 1989]. These studies support our conclusion that the fit horse is capable of attaining a higher degree of exercise intensity, and it is this level of exercise intensity which is responsible for the increased releasability of beta-endorphin that has been noted in trained human athletes.

Cortisol concentrations were significantly increased following all exercise tests, with higher levels noted thirty minutes following the 1000 m exercise tests when compared with the 400 m exercise tests. There was no difference in the cortisol response to exercise between fit and unfit horses following the 400 m exercise test. These results support the conclusion that hormonal release is linked to exercise intensity more than to

the degree of exertional stress. However, increased sampling times may enable detection of a difference in the cortisol response to exercise between fit and unfit horses. Also, adrenocorticotropin concentrations may be a more sensitive indicator of the response to exercise than cortisol, and therefore may be a better alternative.

The correlation between beta-endorphin concentrations and lactate concentrations suggest that earlier reports linking beta-endorphin release with anaerobiosis may in fact be the case [DeMeirleir, *et al.*, 1986; Mougin, *et al.*, 1987]. The injection of lactic acid produces anxiety neurosis in humans [Pitts and McClure, 1972]. Increased lactate levels may therefore be associated with pain and anxiety, as well as exercise intensity, during exercise. Thus, increased beta-endorphin concentrations following exercise may result from increased anxiety or pain induced by elevated lactate levels. The mechanism by which exercise stimulated beta-endorphin release remains unclear. Although it has been suggested that anaerobiosis may be involved, other stimulatory stresses, such as hypoglycemia, or neural pathways may be involved [DeMeirler, *et al.*, 1986]. The increased physical stress of a hot ambient temperature or dehydration has also been noted to increase opioid release [McMurray, *et al.*, 1984], suggesting a relationship exists between exercise stress and the opioid response. Shyu, *et al.* [1982] have suggested that long lasting exercise may produce discharges in muscle afferents which influence central endorphin mechanisms. These investigators concluded that these mechanisms may result in exercise-induced analgesia. It may be through this stimulation of neural pathways that peripheral beta-endorphin release is also effected.

Naloxone is an opiate receptor antagonist which acts primarily at the mu receptor

and is capable of antagonizing the analgesic effects of opiates or endogenous opioids. It is thought to be a pure antagonist with no physiologic actions of its own [Frederickson, 1984]. Naloxone pretreatment resulted in higher beta-endorphin concentrations thirty minutes post exercise in both the fit and unfit horses. These higher levels suggest that naloxone may be blocking autoreceptors for beta-endorphin at the hypothalamic or pituitary level that are involved in regulating beta-endorphin release, thus prolonging the release of beta-endorphin following exercise. Naloxone pretreatment also resulted in higher cortisol concentrations immediately following exercise in the unconditioned but not the conditioned horse. This faster rise in cortisol concentrations following exercise in the unfit naloxone treated horse may again result from blockade of beta-endorphin or opioid receptors or from blockade of receptors at the hypothalamic level which control corticotropin releasing factor (CRF) release. As naloxone is capable of crossing the blood-brain barrier, its interaction with central opioid receptors that regulate pain transmission may increase the pain experienced by the animal. Naloxone has been shown to increase sensitivity to pain, or cause hyperalgesia, following intrathecal injections in rats suggesting that the hyperalgesic effects of naloxone are due to blockade of tonic inhibition by endogenous opioids in the spinal cord [Woolf, 1980]. This theory is confirmed by the significantly decreased pain threshold (or hyperalgesia) following exercise in the unfit naloxone pretreated horse. Therefore, since naloxone pretreatment did not significantly decrease the pain threshold in the fit horse after the 1000 m exercise test, but returned it to baseline, the fit horse did not have the benefit of the increase in pain threshold it normally would have experienced and therefore may have experienced

more pain and discomfort. The negative correlation between beta-endorphin levels and the skin twitch reflex latency noted in unfit naloxone pretreated horses after the 400 m exercise test suggests that increased pain perception may result in increased beta-endorphin and cortisol levels. This is confirmed by the positive correlation between beta-endorphin and cortisol concentrations following exercise tests in both fit and unfit naloxone pretreated horses.

The exercise induced increases in beta-endorphin levels along with increased pain threshold suggest that beta-endorphin may be involved in pain regulation. Beta-endorphin produces analgesia following intracerebroventricular injections and is found both centrally and peripherally [Frederickson and Geary, 1982]. Beta-endorphin that originates in the central nervous system is thought to be involved in pain control while peripherally occurring beta-endorphin may be involved in stress-induced analgesia [Akil, *et al.*, 1984]. Beta-endorphin is generally not thought to cross the blood-brain barrier, although some investigators suggest that opioid peptides have sufficient permeability to exert central effects [Rossier, *et al.*, 1977; Rapoport, *et al.*, 1980]. The role of corticotropin releasing factor (CRF) and glucocorticoids also cannot be eliminated. Corticotropin releasing factor administration produced analgesia in humans and rats [Hargreaves, *et al.*, 1987]. Therefore, exercise produced analgesia may result from the anti-inflammatory effects of increased glucocorticoid levels, resulting from CRF release, or from a direct neural effect of CRF as immunoreactive CRF has been found in the dorsal horn of the spinal cord and the trigeminal nucleus, areas associated with nociception [Schipper, *et al.*, 1983]. It has also been suggested that exercise-induced analgesia may result from muscle afferent

discharges which influence central opioid mechanisms [Shyu, *et al.*, 1982]. Therefore, the analgesia seen following exercise may be due to beta-endorphin or other endogenous opioids originating in the central nervous system, perhaps through stimulation by muscle afferent fibers, or may result from increased CRF levels either at the central or peripheral level. However, a role for peripherally circulating beta-endorphin in exercise-induced analgesia cannot be eliminated.

The hyperalgesia noted after naloxone pretreatment in the unconditioned horse was not seen in the conditioned horse. The fact that hyperalgesia was demonstrated only in the unfit horse suggests that there is a change in opioid receptor number or affinity after conditioning. Up or down regulation of receptors is seen as a physiologic response to changing levels of hormones in the body [Hollenberg, 1985]. Decreased receptor number or decreased affinity of the receptor for beta-endorphin following conditioning would explain the lack of hyperalgesia following exercise in the naloxone pretreated fit horse. If the unfit horse had a higher number of receptors or a higher affinity of the receptors for the ligands, naloxone would either bind to more receptors or bind with greater affinity, and therefore would have a greater effect.

The significance of peripherally released opioid peptides has encouraged research into their role in exercise performance, exercise-induced affective responses such as analgesia, metabolic responses, cardiovascular responses and immune function [Cummings and Wheeler, 1987; Sforzo, 1989]. This study demonstrated the release of beta-endorphin in response to maximal exercise. The pattern of beta-endorphin release suggested that the release was more a function of exercise intensity than exertional stress and may be linked

to anaerobiosis. Increased pain threshold following exercise in the unfit horse following a 400 m exercise test and in the fit horse following a 1000 m exercise test, along with increased heart rates, lactate levels and respiratory rates, demonstrate higher levels of exertional stress during these tests. Peripherally circulating beta-endorphin may be involved with modulating pain perception following exercise, although the role of endogenous opioids which originate in the central nervous system cannot be eliminated. Naloxone pretreatment reversed the analgesia seen following exercise, prolonged beta-endorphin release and accelerated cortisol release. These results suggest that indeed endogenous opioids play a role in modulating the physiologic homeostatic response to exercise. Although some studies suggest that endogenous opioids affect cardiovascular and respiratory responses to exercise [Sforzo, 1989; Grossman, *et al.*, 1984], the results of this study do not support this theory. Naloxone did not significantly alter the cardiovascular or respiratory responses to exercise. However, naloxone is primarily a mu receptor antagonist, while delta and kappa receptors are associated with opioid cardiovascular regulation [Sforzo, 1989]. Also, naloxone appears to control ventilation only at high levels of power output in humans which may not have been reached in this study [Grossman, *et al.*, 1984].

In order to determine whether or not central or peripherally originating endogenous opioid peptides are responsible for the analgesia seen after exercise will require experiments which measure central levels of endogenous opioids. Finally, physical conditioning appears to alter the response of the horse to exercise, thereby decreasing the level of exertional stress. The effects of physical conditioning on the response of the

horse to exercise may involve a number of complex interrelationships. Conditioning may involve adaptations of cardiac, vascular, hormonal, metabolic, neurologic and psychological functions. There may be increased work capacity of the heart, change in muscle fiber type, change in metabolic demands and change in the psychological response to exercise. It is possible that changes seen with conditioning in physiologic functions may be opioid regulated, in part, and therefore are due to changes in opioid receptor number or affinity.

In conclusion, this study demonstrated that beta-endorphin is released in response to exercise in the horse and the response is altered by physical conditioning. The use of naloxone also demonstrated that opioid receptors modulate the physiologic response to exercise, perhaps through interaction of the receptor with circulating beta-endorphin. However, further investigation into the significance of peripherally circulating beta-endorphin is necessary to clarify its role in exercise.

CHAPTER FIVE

CHARACTERIZATION OF BETA-ENDORPHIN RECEPTORS ON EQUINE LYMPHOCYTES

A. INTRODUCTION

Data from current research support the theory of interaction between the central nervous system and the immune system. One finding which supports this theory is the existence of opioid receptors on cells of the immune system. Although a number of investigators have demonstrated binding of beta-endorphin and other opioids to lymphocytes, confusion exists as to the type of opioid receptor existing on lymphocytes. The presence of several types of classical opiate receptors have been reported. Mehrishi and Mills [1983] reported that lymphocytes contained specific opioid receptors of the mu type, as demonstrated by binding of tritiated naloxone to freshly isolated human lymphocytes. Madden, *et al.* [1987] also reported a specific binding site for naloxone on human T lymphocytes. In their study, the bound naloxone was partially displaced by morphine, beta-endorphin, and methionine-enkephalin (met-enkephalin). Recently, Carr *et al.* [1989] have found evidence for both delta and kappa receptors on various cell lines using highly specific ligands. This same group of investigators has also developed an antibody which recognizes opioid receptors on murine splenocytes and neuroblastoma x glioma hybrid cells. This antibody competes with tritiated dihydromorphine as well as beta-endorphin, met-enkephalin, and naloxone for the same binding site on lymphocytes,

which suggests the presence of a classical opioid receptor of the mu or delta class [Carr, *et al.*, 1988a].

The previous studies all suggest the presence of classical opioid receptors on lymphocytes. In contrast, a number of other investigators have been unable to confirm these results and therefore propose that a nonopioid receptor for beta-endorphin exists on lymphocytes. Hazum *et al.* [1979] were unable to demonstrate classical opioid receptors on lymphocytes. In this study, high affinity binding of iodinated beta-endorphin was exhibited only in a transformed human cell line and was of nonopioid specificity. Mendelsohn *et al.* [1985] were unable to demonstrate any specific binding of tritiated naloxone or leucine-enkephalin to freshly isolated human lymphocytes. Several other investigators have reported specific binding of endogenous opioids to lymphocytes, either freshly isolated cells or cell lines, that was not displaced by morphine or naloxone [Ausiello and Roda, 1984; Westphal and Li, 1984; Schweigerer, *et al.*, 1985b; Borboni, *et al.*, 1989]. In addition, Ovadia *et al.* [1989] reported binding of tritiated naloxone to rat lymphocytes which was not displaced by any endogenous opioids.

Due to the conflicting reports, confusion as to the type or class of receptor present on lymphocytes still persists. Many investigators report that beta-endorphin does not appear to bind to classical opioid receptors, although naloxone and other opiates have been shown to bind specifically to lymphocytes. These studies suggest that there may exist two types of sites on lymphocytes, "opioid" and "nonopioid", to which beta-endorphin binds. However, the lack of classical receptor binding studies and the use of cell lines which are not truly representative of circulating lymphocytes, has contributed

to the confusion. Although beta-endorphin receptors on lymphocytes have been examined in humans, mice, and rats, they have not been identified in the horse. As beta-endorphin has been reported to modulate immune function, presumably through interaction with cell surface receptors [Sibinga and Goldstein, 1988], this study attempted to identify and characterize beta-endorphin receptors on equine lymphocytes.

B. MATERIALS AND METHODS

Lymphocyte collection and preparation

Blood was collected from the jugular vein of healthy Thoroughbred horses into 20 ml evacuated siliconized glass tubes containing preservative free heparin[1] at a concentration of 10 U/ml as an anticoagulant. Lymphocytes were isolated using density gradient centrifugation. Whole blood was centrifuged for 10 minutes at 400 x g, the plasma was removed and discarded, and the buffy coat was removed and diluted 1:5 in Hanks' Balanced Salt solution without calcium and magnesium (CMF Hanks)[2] with 20 mM Hepes[3]. Ten milliliters of the cell suspension were layered over 4 ml of Ficoll[4] and centrifuged at 600 x g for 30 minutes. After centrifugation, the upper layer was removed, discarded, and the interface, which contained the mononuclear cells, removed and diluted

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[1]Heparin, preservative free, sodium salt, Grade I: from porcine intestinal mucosa, Sigma Chemical Co., St. Louis, Mo.

[2]Hanks' Balanced Salts without calcium chloride, magnesium sulfate and sodium bicarbonate, Sigma Chemical Co., St. Louis, Mo.

[3]Hepes, sodium salt, Sigma Chemical Co., St. Louis, Mo.

[4]Histopaque-1077, specific gravity 1.077$\pm$0.001, Sigma Diagnostics, St. Louis, Mo.

1:4 in CMF Hanks. The cell suspension was washed three times in CMF Hanks and then resuspended in the assay buffer, Medium 199[5] with Earle's salts, L-glutamine and 25 mM Hepes supplemented with 0.1% bovine serum albumin (BSA)[6], at a concentration of 7.5 x 10^6 cells/ml. Cell viability was assessed by trypan blue dye[7] exclusion. The binding assays were performed within 24 hours of cell collection as longer periods of storage resulted in decreased cell viability.

Determination of optimum time, temperature and pH

Optimum time and temperature were ascertained by incubating 0.1 ml of the cell suspension (3 x 10^6 cells/assay tube) with (3-[^{125}I]iodotyrosyl^{27})beta-endorphin (human)[8], specific activity 2075 Ci/mmol, at a final concentration of 9.9 x 10^{-11} M at 4°C, 25°C and 37°C for 15, 30, 45, 60, 120, 180, and 240 minutes in 12 x 75 polypropylene tubes. The ^{125}I-labeled beta-endorphin used was prepared by iodination of synthetic human beta-endorphin using chloramine-T and sodium [^{125}I]iodide and was purified by high performance liquid chromatography. It is a monoiodinated product, iodinated only at the carboxy-terminal tyrosine (tyrosine residue 27), and therefore is active in receptor binding studies as well as radioimmunoassay. Non-specific binding was determined by incubating the cells and iodinated beta-endorphin in the presence of excess unlabeled beta-

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[5]Medium 199 with Earle's salts, L-glutamine, 25 mM Hepes, without sodium bicarbonate, Sigma Chemical Co., St. Louis, Mo.

[6]Bovine Serum Albumin, Fraction V, Sigma Chemical Co., St. Louis, Mo.

[7]Direct Blue 15, Sigma Chemical Co., St. Louis, Mo.

[8](3-[^{125}I]iodotyrosyl^{27})beta-endorphin (human), Amersham Corp., Arlington Heights, Il.

endorphin[9] at a final concentration of 3.6×10^{-6} M. All dilutions and incubations were performed in assay buffer, pH 7.4. Incubation was terminated at the appropriate time by removing duplicate 170 µl samples of well mixed cell suspension from each tube and adding to ice cold microfuge tubes[10] containing 100 µl of dibutyl phthalate[11] and 100 µl of assay buffer. The microfuge tubes were then centrifuged in a microcentrifuge[12] for 2 minutes, the supernatants were aspirated and the tips of the microfuge tubes, which contained the cell pellet, were cut off and placed in 12 x 75 glass tubes. The tubes were then counted for 5 minutes in a gamma scintillation counter.

Optimum temperature and pH were assessed based on the optimum times determined in the previous experiment. Again, 3×10^6 cells/assay tube were incubated with 9.9×10^{-11} M iodinated beta-endorphin for 30 minutes at 37°C, 1 and 4 hours at 25°C and 1 hour at 4°C in assay buffer at pH's of 7.0, 7.4, 7.6, 8.0 and 8.4. Nonspecific binding was again determined by incubating the cells and radiolabeled beta-endorphin in the presence of excess unlabeled beta-endorphin (3.6×10^{-6} M). The incubation was terminated and analyzed as described previously.

Determination of saturability

The saturability of the receptors was analyzed by setting up a saturation isotherm. This assay allows one to analyze the characteristics of the binding as a function of

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[9]Beta-endorphin, human; beta-lipotropin (61-91), human; Bachem, Inc., Torrance, Ca.

[10]Eppendorf Micro test tubes, 400 ul, polypropylene, Brinkmann Instruments, Inc., Westburg, N.Y.

[11]Dibutyl phthalate, Sigma Chemical Co., St. Louis, Mo.

[12]Abbott microcentrifuge, Model LN 9527-01, Abbott Laboratories, Irving, Texas.

increasing concentrations of radioligand. In this assay, radiolabeled beta-endorphin (specific activity 1800 Ci/mmol) was added to 3 x 10^6 cells/assay tube to give final concentrations of 42.5, 81.7, 200.3, 432.0, 1017.7, 1142.9, 1430.2, 2648.6, 2827.8, and 5117.4 pM. The cells and the radiolabeled beta-endorphin were incubated with and without excess unlabeled beta-endorphin (3.6 x 10^{-6} M) for 4 hours at 25°C in assay buffer, pH 8.0. The binding found in the presence of excess unlabeled beta-endorphin was determined to be nonspecific binding. The incubation was terminated by centrifugation in a microfuge and subsequent analysis by a gamma scintillation counter as described previously.

Determination of specificity

The specificity of the receptor for beta-endorphin was determined by analyzing the ability of various ligands to displace the radioligand from the receptor. Equine lymphocytes (3 x 10^6 cells/assay tube) were incubated with iodinated human beta-endorphin (specific activity 1800 Ci/mmol) at a final concentration of 3.2 x 10^{-10} M and different ligands at concentrations ranging from 1 pM to 42 μM for 4 hours at 25°C in assay buffer, pH 8.0. The ligands and concentrations used were: human beta-endorphin, equine beta-endorphin,[13] naloxone,[14] and [D-Ala^2]-met-enkephalinamide[15] at concentrations of .001, .01, .1, 1, 10, 100, and 1000 nM; fentanyl[16] at concentrations of

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[13]Beta-endorphin (horse), Peninsula Laboratories, Inc., Belmont, Calif.

[14]Naloxone hydrochloride, Sigma Chemical Co., St. Louis, Mo.

[15][D-Ala^2]-Met-Enkephalinamide, Peninsula Laboratories, Inc., Belmont, Calif.

[16]Fentanyl citrate, McNeil Pharmaceuticals, Inc., Spring House, Penn.

.001, .01, .1, 1, 10, 100, 1000 and 10000 nM; ethylketocyclazocine[17] at concentrations of .0835, .835, 8.35, 83.5, 835, 8350, and 41800 nM; and U50,488H[18] at concentrations of .068, .677, 6.771, 67.71, 677.05, 6770.5 and 33852 nM. The incubation was terminated by addition of duplicate 170 µl samples from each assay tube to ice-cold 400 µl microfuge tubes containing 100 µl of assay buffer and 100 µl of dibutyl phthalate. The microfuge tubes were then centrifuged for 2 minutes in a microcentrifuge, the supernatants aspirated, and tips of the microfuge tubes cut and placed in 12 X 75 glass tubes. The tubes were counted for 5 minutes in a gamma scintillation counter. Nonspecific binding was determined as the amount of binding noted in the presence of 1000 nM of equine beta-endorphin.

Radioligand degradation

To minimize radioligand degradation, the lyophilized iodinated peptide was reconstituted in distilled water at a concentration of 0.1 µCi/µl. In order to prevent damage by repeated freezing and thawing, the radioligand was then aliquoted in 50 µl aliquots, enough for one day's experiments, in polypropylene vials and stored at -20°C. The radioligand was used by the expiration date given by the manufacturer which was within 28 days of receipt. According to stability trials carried out by the manufacturer, the decomposition of the reconstituted radioligand should not exceed 5% by the expiration date under these conditions.

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[17]Ethylketocyclazocine methanesulfonate, Sterling-Winthrop Pharmaceutical Co., New York, N.Y.

[18]U50,488H, Upjohn Co., Kalamazoo, Michigan.

C. RESULTS

1. Effects of time, temperature and pH

The analysis of the effects of time and temperature on the binding of beta-endorphin to equine lymphocytes revealed optimum specific binding at 25°C (Figure 21). Cell viability was greater than 90% for all assays as determined by the trypan blue exclusion test. Specific binding was calculated by subtracting the counts per minute (cpm) of the nonspecific binding tubes from the cpm's of the total binding tubes. Specific binding represents the actual binding of the radioligand to the receptor site as opposed to nonspecific binding sites, such as trapped radioactivity in the pellet or binding to the incubation tubes. Binding at 37°C reached equilibrium by 15 to 30 minutes. Binding at 25°C appeared to reach equilibrium between one and four hours, although there was considerable assay variability. Binding at 4°C reached equilibrium by one hour but binding began to deteriorate after 3 hours.

Based on these observations, the effects of time, temperature and pH were analyzed. Several time and temperature combinations were considered over a range of pH's for optimum specific binding. Specific binding was greatest at 25°C, but assay variation made the determination of equilibrium difficult. Therefore, binding at 25°C was analyzed at both 1 and 4 hours of incubation over a range of pH values. Binding at 37°C plateaued by 15 minutes and remained stable for up to one hour. Therefore, an incubation time of 30 minutes was chosen for pH analysis. The specific binding at 4°C plateaued by one hour and therefore was analyzed for effect of pH at this incubation time. Incubation over a range of pH's from 7.0 to 8.4 revealed that at 4°C and 37°C, optimum

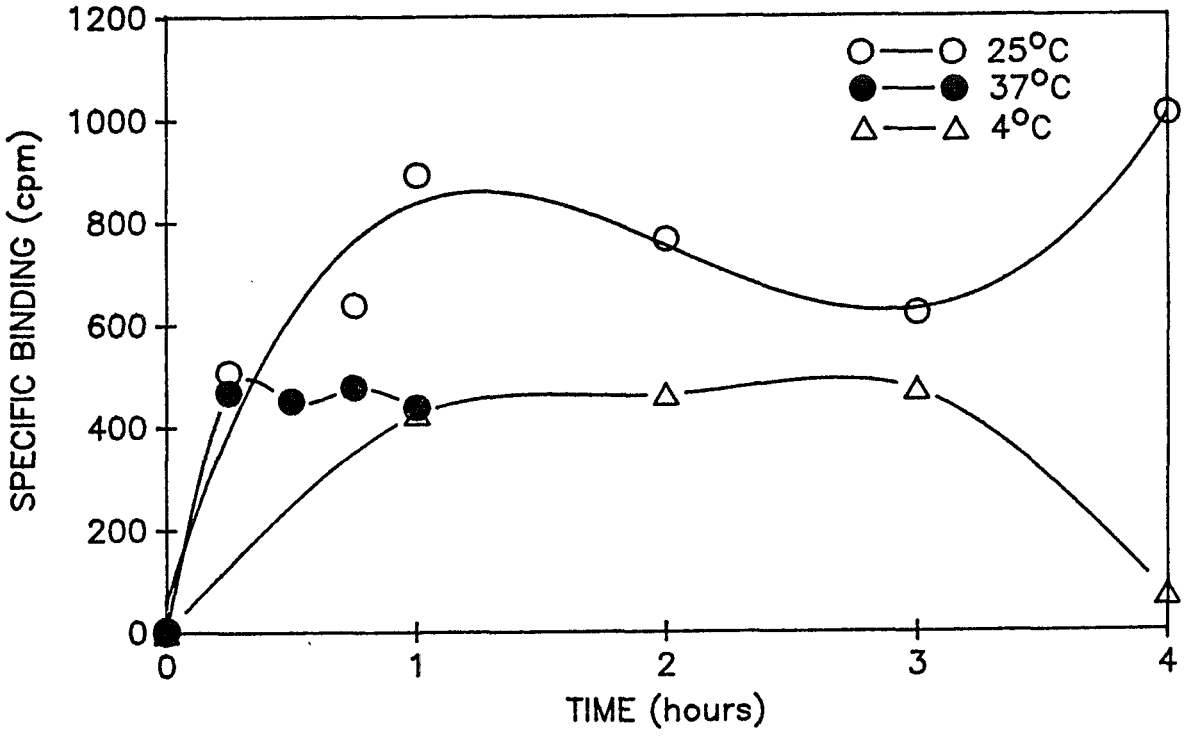

Figure 21. Rate of binding of [^{125}I]beta-endorphin to equine lymphocytes at a pH of 7.4 at various temperatures.

specific binding was noted at a pH of 7.6 (Figure 22). At 25°C after one hour of incubation, the greatest binding was noted at pH 7.6. However, after four hours of incubation at a pH of 8.0, there was a dramatic increase in specific binding at 25°C. The effects of pH on binding were not dramatic at most time and temperature combinations, with the level of specific binding approximately the same for all with the one exception. This experiment was repeated and confirmed to ensure that the dramatic increase in binding at 25°C at pH 8.0 after 4 hours incubation was not an artifact, and was found to be reproducible. It is possible that this combination of pH, time and temperature somehow stabilizes the receptor, perhaps by preventing receptor internalization. Therefore, the incubation conditions for future experiments were chosen as 25°C for 4 hours at a pH of 8.0.

2. Receptor saturability

The data from the saturation isotherm were analyzed in three ways. First, the data were plotted in the standard format of concentration of free radioligand versus bound radioligand (Figure 23). Analysis of the linear saturation isotherm reveals an apparent saturation of the receptor as demonstrated by the plateau achieved in the specific binding curve. Specific binding was calculated by subtracting the nonspecific binding from the total binding. Nonspecific binding increased in a linear manner (r=0.94490), which suggests that it was not saturable, as would be expected. Unfortunately, a linear saturation isotherm does not always truly demonstrate saturability, and therefore requires further analysis. A logarithmic transformation of the data, as seen in Figure 24, demonstrated that saturation of the receptors was achieved, although not at the

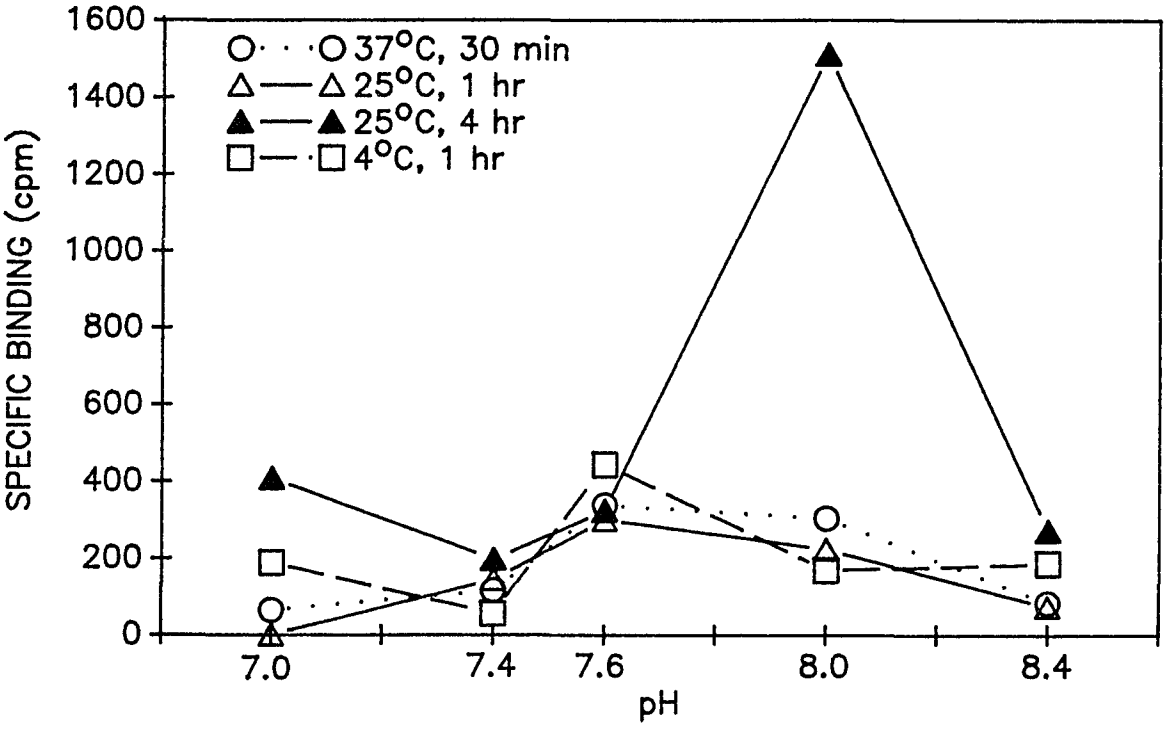

Figure 22. Specific binding of [^{125}I]beta-endorphin to equine lymphocytes over a range of pH's.

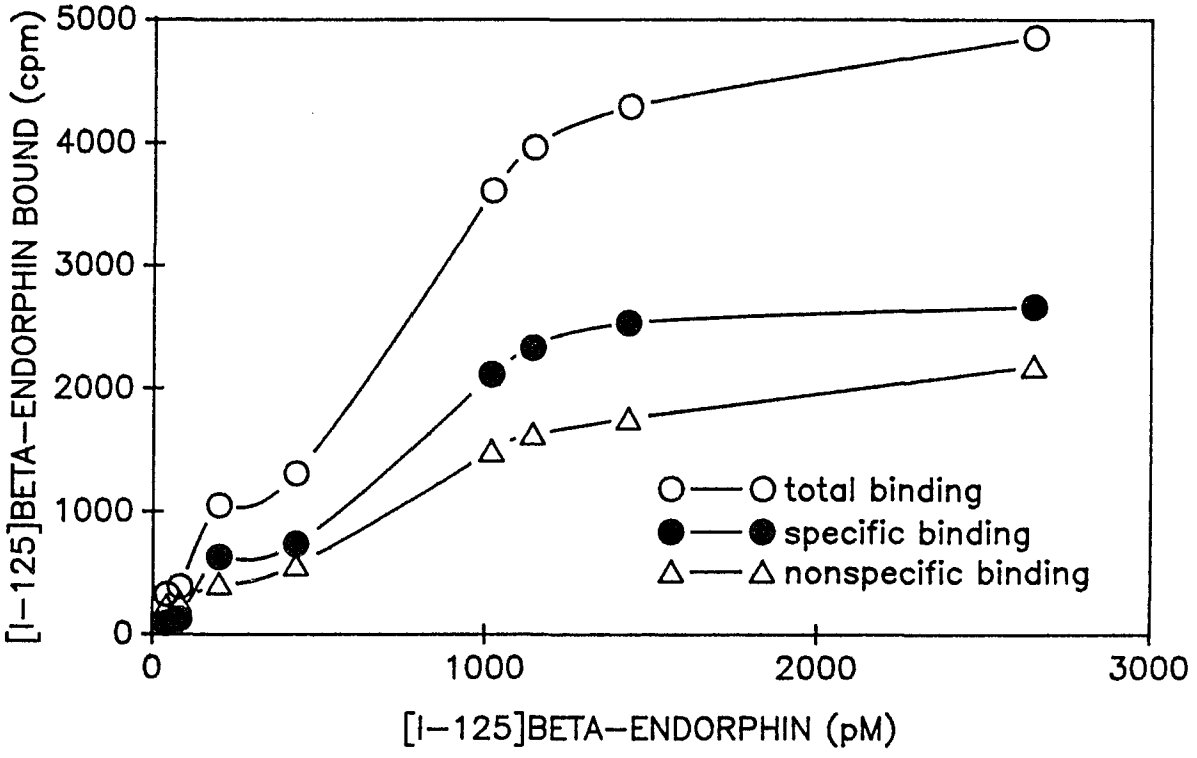

Figure 23. Saturation isotherm of equine lymphocyte beta-endorphin receptors plotted on a linear scale.

concentrations at which saturation seemed to occur in the linear plot. In the linear plot, saturation appeared to occur at radioligand concentrations of 1.5 nM, while in the logarithmic transformation, saturation did not occur until radioligand concentrations of 2.0 nM or greater were reached.

3. Estimation of Binding Parameters

A Scatchard/Rosenthal linear transformation of the data was performed in order to obtain estimates of the binding parameters: the dissociation constant or K_d, and the total number of binding sites or B_{max} (Figure 25). In a Scatchard plot, linear regression analysis of the points yields a y-intercept and the slope, from which the binding parameters can be calculated. The dissociation constant (K_d) is calculated from the slope (K_d = -1/slope). Analysis of the data revealed a dissociation constant of 17.2 pM or 1.72 x 10^{-11} M for beta-endorphin receptors on equine lymphocytes. Using the y-intercept and the slope, the x-intercept can be calculated. The x-intercept is the total number of binding sites or B_{max}. Based on these calculations, the x-intercept or B_{max} was determined to be 0.538 fmoles/10^6 cells or 5.38 x 10^{-16} moles/10^6 cells. The correlation coefficient for the linear regression was 0.68836 ($P<0.01$). Further analysis of the saturation binding data was performed using the LUNDON-1 computer program. This computer program utilized a nonlinear least squares curve regression analysis and employed progressive curve fitting with concurrent statistical analysis enabling analysis of goodness of fit between successively more complicated models [Lundeen and Gordon, 1986]. Analysis of the saturation binding data using the LUNDON-1 program revealed that a one-site model best fit the data. Therefore, a single slope was obtained from the linear transformation

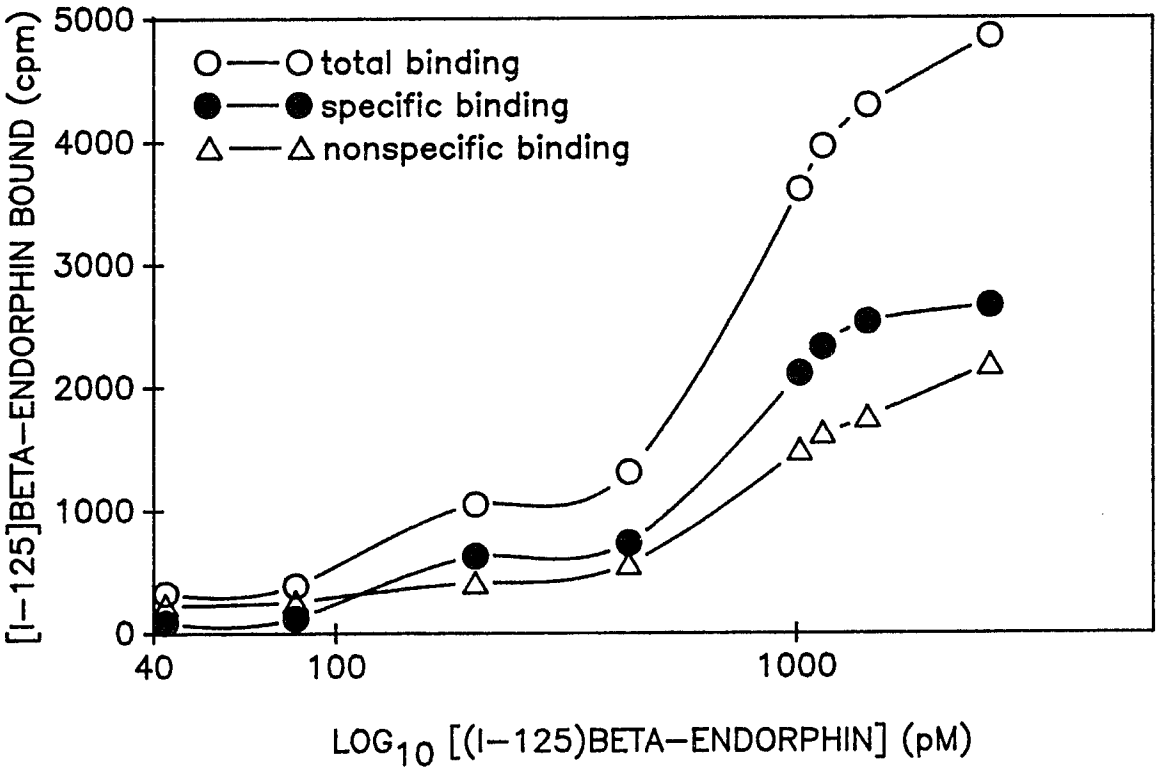

Figure 24. Saturation isotherm of equine lymphocyte beta-endorphin receptors plotted on a logarithmic scale.

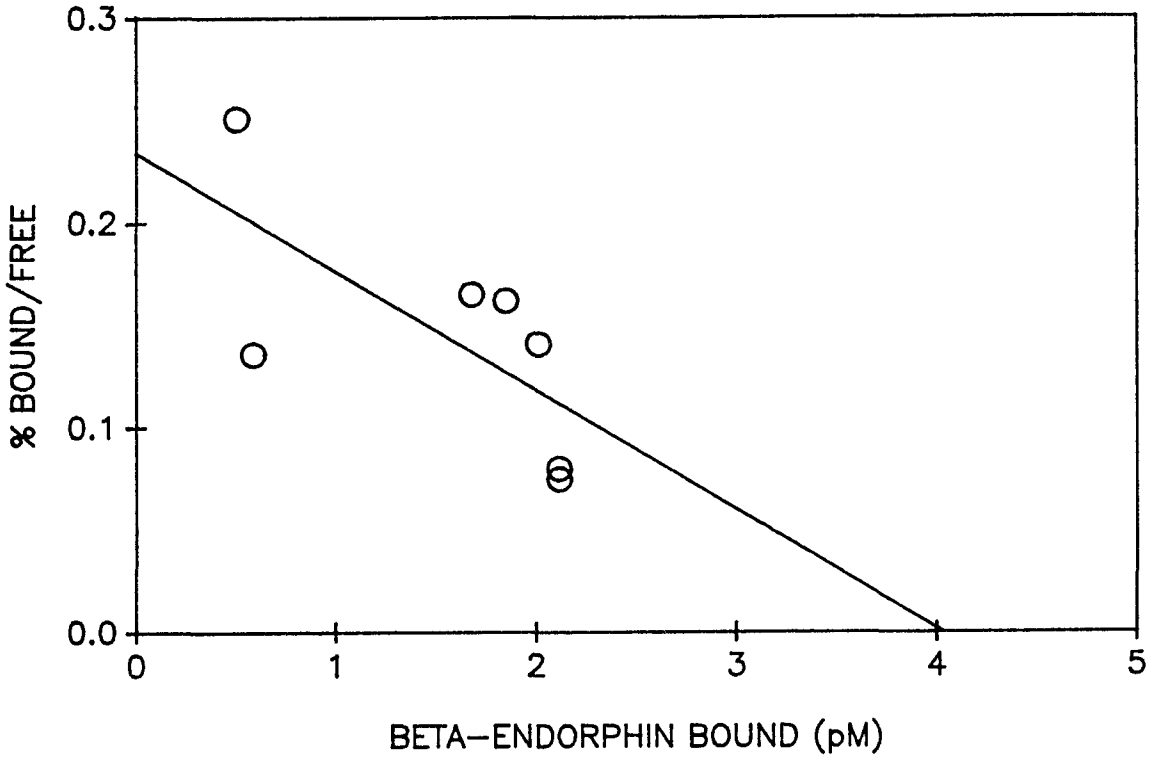

Figure 25. Scatchard/Rosenthal analysis of saturation binding data of beta-endorphin receptors on equine lymphocytes.

suggesting that a single receptor population exists. If more than one receptor population existed or if the ligand-receptor interaction did not follow the law of mass action, a curvilinear plot with multiple slopes would result.

4. Receptor specificity

The specificity of the receptor for beta-endorphin was assessed by performing competition-inhibition curves of human iodinated beta-endorphin with various opioid ligands. The ability of these ligands to displace the radioligand from the receptor is shown in Figure 26. Highest potency at the receptor was observed with equine beta-endorphin and [D-Ala^2]-met-enkephalinamide. Human beta-endorphin, naloxone and fentanyl were also able to displace the radioligand from the receptor although greater concentrations were required. Ethylketocyclazocine and U50,488H were unable to displace radiolabeled human beta-endorphin from the receptor at meaningful concentrations suggesting that they possess little or no activity at the receptor. From the overall "shape" of a competition curve, it can be determined whether or not a competitor is interacting with the receptor by simple mass action law or with greater complexity. If the ligand-receptor interaction is a simple bimolecular reaction, the competition curve for the competitor will proceed from 10% to 90% competition over an 81-fold concentration range of the competitor. Therefore curves which do not exhibit "normal steepness" are said to result from complex ligand-receptor interactions [Limbird, 1986]. All competitors in this study required approximately a 1000-fold concentration range to displace from 10% to 90% of the bound radioligand. Therefore, the ligand-receptor

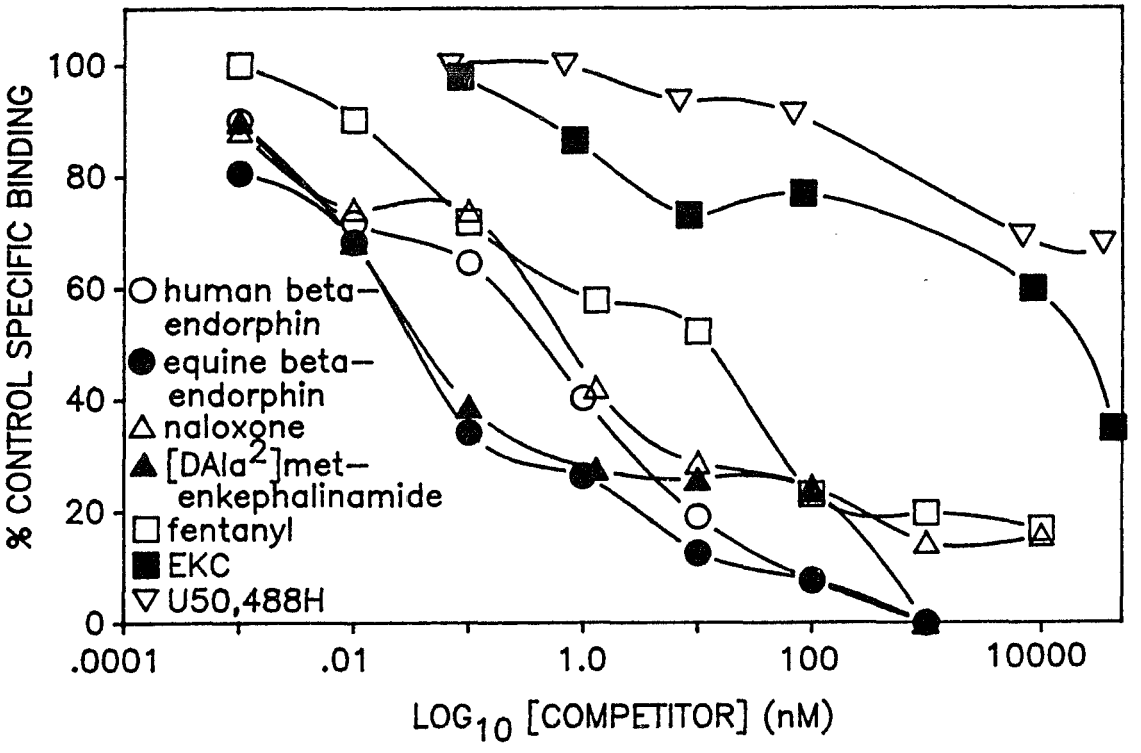

Figure 26. Competition-inhibition curves for various ligands with beta-endorphin receptors on equine lymphocytes.

interactions of the competitors could be described as complex.

In order to compare relative potencies of the ligands, the concentration of the ligand that inhibited the binding of the radioligand by 50% (IC_{50}) was calculated from the competition-inhibition curves. The results are displayed in Table 9. Analysis of the competition-inhibition curves through calculation of IC_{50}'s emphasizes the results which can be obtained visually from the graphs. Equine beta-endorphin has the highest potency, which suggests that this is the endogenous ligand for the receptor. Interestingly, the met-enkephalin analog, a delta opiate receptor ligand, had greater potency for the receptor than did human beta-endorphin and the mu opiate receptor ligands, naloxone and fentanyl. The kappa receptor ligands, ethylketacyclazocine and U50,488H, had little or no activity at this receptor.

D. DISCUSSION

This study demonstrated that equine lymphocytes possess opioid binding sites, as shown with iodinated human beta-endorphin. The binding takes place in a saturable and specific manner which is time, temperature and pH dependent. Optimum binding conditions were demonstrated with incubations at 25°C, pH 8.0 for 4 hours. The assay conditions which were found to be optimum for specific binding are not representative of the physiologic environment of lymphocytes, but may be necessary in order to accurately measure binding to receptors on functional whole cells as opposed to cell membrane fragments. Experiments by Schweigerer *et al.* [1985a] suggest that the receptor-ligand complex is internalized via a high affinity binding site on murine

TABLE 9. Relative Potencies Derived From Competition-Inhibition Curves

	IC_{50}	RELATIVE POTENCY
Equine beta-endorphin	35 pM	1.0
[D-Ala^2]-met-enkephalinamide	40 pM	1.1
Human beta-endorphin	450 pM	12.8
Naloxone	600 pM	17.1
Fentanyl	10 nM	286.0
Ethylketocyclazocine	>100 nM	>3000.0
U50-488H	>100 nM	>3000.0

thymoma cells. If internalization occurs in equine lymphocytes, detection of specific binding would be very difficult, as the radioactivity associated with the internalized beta-endorphin would appear to be nonspecific binding. Therefore, it may be that the combination of a relatively high pH at a lower temperature than seen physiologically may somehow stabilize the receptor so that internalization does not occur. This theory would explain the high nonspecific binding in relation to specific binding which was noted at lower pH's and higher temperatures. The use of a monoiodinated radioligand also increased the ability to detect a receptor on equine lymphocytes. Earlier work on this project, with a less highly purified product, iodinated at one or both tyrosine residues, did not reveal any specific binding, only nonspecific binding to equine lymphocytes. This may have resulted from denaturation of the peptide resulting in loss of activity at the receptor, or from iodination at the N-terminal tyrosine, which was in the binding region of the molecule. The use of iodinated products which were iodinated on the N-terminal tyrosine may explain why some investigators failed to detect opioid binding sites on lymphocytes in other species.

There are certain criteria which must be met in order to establish that there is binding to a physiologically relevant receptor. The binding of the ligand should be saturable since a finite number of receptors are presumed to exist, and the specificity of various ligands at the receptor should parallel the specificity of the ligands *in vivo*. Finally, the kinetics of the binding should be consistent with the time-course of the biological effect elicited by the ligand. In the case of equine beta-endorphin receptors, the binding assay revealed association of the radioligand to the receptor in a time-

dependent manner. Whether or not this mimics the time-course of the biological effect can not be determined as the biological effect has not been properly evaluated, and consequently there is not enough consistent data on which to base such an evaluation.

Analysis of the saturation isotherm data revealed a saturable site which was confirmed by a logarithmic transformation of the data. A Scatchard/Rosenthal linear transformation of the saturation binding data enabled estimation of the binding parameters. The K_d was 17.2 pM or 1.72 x 10^{-11} M and B_{max} was 0.538 fmoles/10^6 cells. The dissociation constant is consistent with biological levels of beta-endorphin in the plasma in the horse. Earlier work [Chapter 4] has shown basal circulating levels of beta-endorphin in the horse to range from 2-40 pmoles/L. Other investigators have reported dissociation constants in the nanomolar range in humans which seems unlikely as plasma levels in humans are similar to those in horses [Sforzo, 1989; Madden, *et al.*, 1987]. If the dissociation constant was in the nanomolar range, then plasma levels would have to reach 100 to 1000 times the basal circulating levels in order to elicit an effect. The fact that a linear Scatchard plot was obtained allows one to assume that a fairly accurate estimation of the true K_d has been made [Limbird, 1986]. Based on these observations, it seems reasonable to assume that beta-endorphin binds in a saturable manner to equine lymphocyte with a K_d of 17.2 pM. The B_{max} values usually obtained from Scatchard/Rosenthal plots, represent the total number of binding sites. However, if n=1, i.e. one binding site, then B_{max} represents the total receptor concentration. Since the Scatchard plot in this experiment was linear, it can be assumed that only one binding site exists, and the B_{max} value of 0.538 fmoles/10^6 cells represents the total receptor

concentration. Nevertheless, this number has little meaning in itself since the receptor has not been isolated. Without isolation of the receptor, and determination of its molecular weight, the actual number of receptors can not calculated. Therefore, the B_{max} value obtained in this study represent an approximation of the number of moles of beta-endorphin which bind to the cells.

Competitive binding analysis revealed that the receptor on equine lymphocytes binds opioids which have affinity for the mu and delta classes of opioid receptors. Beta-endorphin binds with equal affinity to mu and delta receptors [Sforzo, 1989]. [DAla^2]-met-enkaphalinamide is a met-enkephalin analog with increased resistance to enzymatic degradation, which binds preferentially to the delta opioid receptor [Pasternak and Wood, 1986]. Naloxone and fentanyl both bind preferentially to the mu opioid receptor [Sforzo, 1989; Jaffe and Martin, 1985]. Ethylketocyclazocine (EKC) and U50,488H are selective kappa opioid receptor agonists [VonVoigtlander, *et al.*, 1983; Pert, *et al.*, 1976]. Equine beta-endorphin and [D-Ala^2]-met-enkephalinamide displaced the radioligand at the lowest doses, suggesting that they possess the greatest affinity for the receptor. Naloxone, human beta-endorphin and fentanyl were also able to displace the radioligand, but required higher concentrations. The kappa agonists, EKC and U50,488H were unable to displace the radioligand effectively, suggesting the lack of a specific kappa binding site on equine lymphocytes.

The results suggest that the receptor present on equine lymphocytes is of the mu or delta class. The high affinity of the enkephalin analog suggests that the receptor may be of the delta class. Reports of a common site with very high affinity for both

enkephalins and mu receptor agonists such as morphine, termed the mu_1 site, also imply that this receptor may in fact be a mu_1 receptor site [Pasternak and Wood, 1986]. Most recently, evidence for a mu/delta opioid receptor complex with interacting mu and delta binding sites has been demonstrated in rat striatal membranes [Schoffelmeer, *et al.*, 1990]. This theory perhaps best explains the data reported from the competition curves. The slope of the competition curves suggested that the competitors interacted with the receptor in a manner more complex than that described by the law of mass action. In other words, rather than a single ligand interacting with a single receptor population in a reversible manner, the ligands may be interacting with more than one receptor population, or interacting with a receptor with different affinity sites. An interacting mu/delta receptor complex would result in a more complex ligand-receptor interaction which would in turn result in competition curves which do not exhibit "normal steepness". The interacting mu/delta receptor complex would also explain the ability of both beta-endorphin and the enkephalin analog to bind to the site with such high affinity. If the receptor site was of the delta class or the mu_1 subtype, it would seem that the competition curves would have demonstrated "normal steepness". However, the Scatchard analysis of the saturation isotherm did not demonstrate a complex receptor-ligand interaction, but rather a simple interaction of a single ligand with a single population of receptors. Due to technical and cost constraints, the saturation isotherm was performed with only ten concentrations of radioligand. Many saturation isotherms are performed with twenty or more concentrations of radioligand [Limbird, 1986]. Therefore, increased number of radioligand concentrations in the saturation isotherm may have revealed a more complex interaction

between the radioligand and the receptor, thus eliminating the discrepancy between the saturation and competition data.

In conclusion, an opioid receptor for beta-endorphin has been identified on equine lymphocytes which is both saturable and specific. It appears to be of the mu or delta class of opioid receptors, although results from the competition-inhibition curves suggest it may be a mu/delta receptor complex. The existence of such a receptor on equine lymphocytes coupled with reports of beta-endorphin modulation of lymphocyte function, suggest a role for beta-endorphin and other endogenous opioids in equine immune function.

CHAPTER SIX

CHANGES IN BETA-ENDORPHIN BINDING PARAMETERS OF EQUINE LYMPHOCYTES: EFFECTS OF EXERCISE AND PHYSICAL CONDITIONING

A. INTRODUCTION

Plasma beta-endorphin levels increase significantly in response to moderately high intensity exercise (i.e. 80% VO_{2max} or greater) or maximal exercise intensity (i.e. graded exercise test to exhaustion) in humans [Gambert, *et al.*, 1981; Goldfarb, *et al.*, 1987]. While some investigators report augmented beta-endorphin release following high intensity exercise in trained human athletes as opposed to untrained or sedentary individuals, other studies contradict these results [Carr, *et al.*, 1981; Howlett, *et al.*, 1984]. These studies have raised the question as to whether the absolute exercise intensity which only trained athletes are able to attain is responsible for the increased beta-endorphin levels noted in most studies. Although increased beta-endorphin levels have been reported following exercise in the horse, the effects of training and exercise intensity have not been examined [Evans, *et al.*, 1985; Li and Chen, 1987]. Recent work in our laboratory has demonstrated significantly increased levels of beta-endorphin following maximal intensity exercise [Hamra, *et al.*, 1987]. Training did not augment beta-endorphin release, although increased exercise intensity, which was attainable only by the trained horses, resulted in significantly higher beta-endorphin levels [Chapter 4].

Receptors for beta-endorphin and other opioid peptides have been identified on human and murine leukocytes. A number of in vitro studies have demonstrated the ability

of opioid peptides to modulate immune function, such as lymphocyte proliferative responses and natural killer cell activity [Sibinga and Goldstein, 1988]. Therefore, it appears that endogenous opioids, which are released in response to stress, such as exercise, can interact with cells of the immune system and thereby modulate immune function. Although considerable work has been done in humans, mice, and rats, this issue has not been addressed in the horse. Previous work in our laboratory has demonstrated highly specific, saturable opioid receptors for beta-endorphin on equine lymphocytes [Chapter 5]. Therefore, we have established that beta-endorphin is released in response to exercise, that this response changes with physical conditioning and that equine lymphocytes possess a receptor for beta-endorphin. These results suggest a role for beta-endorphin in equine immune function.

Recently exercise, as a form of exertional stress, has been reported to modify immune function. Exercise has been shown to modify the proportions of lymphocyte subsets, enhance natural killer cell activity and suppress lymphocyte blastogenesis in response to mitogens in humans [Hedfors, *et al.*, 1976; Keast, *et al.*, 1988]. One study in horses demonstrated a significant suppression of the blastogenic response to the mitogens Concanavalin A and phytohemagglutinin 30 minutes after treadmill exercise to fatigue [Kurcz, *et al.*, 1988]. These studies suggest that exercise modulates equine immune function, perhaps through interaction of beta-endorphin with receptors on equine lymphocytes. In this study, the effects of exercise and physical conditioning on beta-endorphin lymphocyte receptor binding parameters were evaluated in order to clarify the role of beta-endorphin and exercise in equine immune function.

B. MATERIALS AND METHODS

Horses

Fifteen healthy Thoroughbred horses, five males and 10 females with a mean age of 5.9±1.5 years, were used in evaluating the effect of exercise on binding parameters in trained horses. All horses had received previous racing and/or training experience but had not been raced or trained for at least six months prior to the study. The horses were subsequently trained on a high speed treadmill[1] using interval training techniques [Harkins, *et al.*, 1990].

Twelve healthy Thoroughbred horses, seven males and five females with a mean age of 6.7±1.7 years, were used in evaluating binding parameters in a population of sedentary horses. Four horses had not been raced or trained for at least six months, while eight horses had been detrained from the first study for 12 or more weeks.

Exercise testing and training regimen

Horses were conditioned using an interval training program for 20 weeks. The training regimen is detailed in Table 10. Horses received five days of training and 2 days off per week during the entire training period. Interval training (IT) was started during week 13 and continued through week 20. Interval training was performed twice a week with 3-4 days between training sessions and a more conventional exercise routine performed three days/week. The conventional exercise routine consisted of a walk at 1.5 m/s for 1 min (90 m), a trot at 3.5 m/s for 2 min (420 m), a gallop at 9.0 m/s for 6 min

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[1]Mustang 2200 High Speed Treadmill, Kagra International Inc., Fahrwangen, Switz.

TABLE 10. Training Schedule

WEEK		TOTAL DISTANCE
1-3	180m-2 min walk, followed by a trot increasing from 3.5-4.5 m/s in speed from weeks 1-3, followed by a 90m-1 min walk	2370-3070m
4-12	90m-1min walk, then 420m-2min trot, followed by a gallop increasing from 7.6 m/s to 9.0 m/s over the 8 week period, followed by a 630m trot	3420-6340m
13-14	90m walk, 420m trot warmup, gallop at a 7% incline at speeds increasing from 9.0m/s to 10.5 m/s over the 2 weeks, followed by a 700-800m trot at 3.5m/s as a rest period	5210m
15-18	90m walk, 420m trot warmup, gallop at 7% incline at speeds increasing from 11.0 m/s to 13.0 m/sec over the 4 weeks, followed by a 600m trot at 3.5 m/s as a rest period	4710m
19-20	90m walk, 420m trot warmup, gallop at 7% incline at speeds increasing from 13.0 m/s to 14.6 m/s over the 2 weeks, followed by a 600m trot at 3.5 m/s as a rest period	5310m

(3200 m), and a warm-down at 3.5 m/s for 3 min (630 m) for a total distance of 4340 m at a 0% treadmill inclination.

Interval training consisted of progressively faster heats, performed at a 7% incline, separated by rest periods of a trot at 3.5 m/s at a 0% incline lasting 2-4 min depending on the time required for the heart rate to return to a threshold level of 110 beats/min. During weeks 13 and 14, two heats were run with speeds beginning at 9.0 m/s and progressively increasing to 10.5 m/s. The number of heats increased to 3 during weeks 14-18 and then increased to 4 during weeks 19-20. As the number of heats increased, the distance was shortened, and the speed was progressively increased. By week 20, the speed had increased from 9.0 m/s to 14.6 m/s, and the distance was reduced from 1600 m/heat to 600 m/heat.

The exercise tests consisted of one interval training session during weeks 18, 19, 20. Six horses were tested during an interval training session in week 18, three during week 19 and six during week 20. Although the exercise protocols differed slightly depending on the week in which the horse was tested, all horses reached a near maximal exercise intensity. The protocol for the exercise tests is detailed in Table 11. All gallops in the exercise tests were performed on a 7% incline, with each heat separated by a rest period. During the rest periods, the horses were trotted at a 0% incline. Heart rates were measured by an on-board heart rate monitor[2] which was supplied with the treadmill. The heart rate was transmitted by telemetry from two electrodes attached to a girth strap to two receivers mounted on the treadmill panel. The heart rate was automatically relayed

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[2]Hippocard, Bioengineering of Zurich, Kagra International, Inc., Fahrwangen, Switz.

TABLE 11. Exercise Test Protocols

Week	Heat	Warm-up	Gallop	Warm-down	Total Distance
18	1	90m walk, 420m trot	12.5 m/s, 800m	3.5 m/s, 600m	4710m
	2		12.8 m/s, 800m	3.5 m/s, 600m	
	3		13.0 m/s, 800m	3.5 m/s, 600m	
19	1	90m walk, 420m trot	13.0 m/s, 600m	3.5 m/s, 600m	5310m
	2		13.3 m/s, 600m	3.5 m/s, 600m	
	3		13.6 m/s, 600m	3.5 m/s, 600m	
	4		13.9 m/s, 600m	3.5 m/s, 600m	
20	1	90m walk, 420m trot	13.7 m/s, 600m	3.5 m/s, 600m	5310m
	2		14.0 m/s, 600m	3.5 m/s, 600m	
	3		14.3 m/s, 600m	3.5 m/s, 600m	
	4		14.6 m/s, 600m	3.5 m/s, 600m	

every 5 seconds to the display panel above the treadmill, and was recorded manually every 15 seconds Peak heart rate was reported as an average of the peak heart rates attained during each heat for each exercise test. Blood samples for beta-endorphin levels, lactate levels and radioligand binding assays were drawn from the jugular vein prior to exercise at rest in the stalls, in order to avoid excitement, and immediately upon completion of the exercise test.

Sample Analysis

Blood samples for plasma beta-endorphin concentrations were collected into 7 ml evacuated siliconized glass tubes containing EDTA as an anticoagulant. Blood samples for lactate concentrations were collected into 4 ml evacuated glass tubes containing sodium fluoride as a preservative. The samples were kept on ice until they could be centrifuged, no longer than 1.5 hours. The samples were then centrifuged and the plasma or serum removed and frozen at -20°C until the assays could be performed. Lactate concentrations were measured using a lactate analyzer[3] which measures levels of L-lactate in serum, plasma or whole blood. Plasma beta-endorphin concentrations were measured using a commercially available radioimmunoassay kit (For detailed procedures and validation, refer to Chapter 3).

Radioligand Binding Assay

Binding parameters for beta-endorphin receptors on equine lymphocytes were analyzed by performing Scatchard analysis of competition-inhibition curves. Competition-inhibition curves were performed on each trained horse before (at rest) and after exercise,

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[3]Lactate analyzer, Model 23L, YSI, Inc., Yellow Springs, Ohio.

and on each sedentary horse at rest. Blood samples for the radioligand binding assays were collected into 20 ml evacuated siliconized glass tubes containing preservative free heparin at a concentration of 10 U/ml as an anticoagulant. Lymphocytes were isolated using density gradient centrifugation as described in Chapter 5. After the cells were isolated and washed, they were suspended in assay buffer, Medium 199 with Earle's salts, L-glutamine and 25 mM Hepes supplemented with 0.1% bovine serum albumin, pH 8.0, at a concentration of 7.5 x 10^6 cells/ml. Cell viability was assessed by trypan dye exclusion. The binding assays were performed within 24 hours of cell collection as longer periods of storage resulted in decreased cell viability.

Optimal assay conditions were determined previously [Chapter 5]. Equine lymphocytes (3 x 10^6 cells/assay tube) were incubated with (3-[^{125}I]iodotyrosyl^{27})beta-endorphin (human) at a final concentration of 3.2 x 10^{-10} M and equine beta-endorphin at concentrations of .001, .01, .1, 1, 10, 100, 1000, and 5000 nM in assay buffer for 4 hours at 25°C in a total volume of 400 µl in 12 x 75 polypropylene tubes. The incubation was terminated by addition of duplicate 170 µl samples from each assay tube to ice-cold 400 µl microfuge tubes containing 100 µl assay buffer and 100 µl of dibutyl phthalate. The microfuge tubes were then centrifuged for 2 minutes in a microcentrifuge, the supernatants aspirated, and the tips, which contained the pellets, were cut and placed in 12 x 75 glass tubes. The tubes were then counted for 5 minutes in a gamma scintillation counter. Nonspecific binding was determined by the amount of binding present at excess unlabeled concentrations of equine beta-endorphin; that is, binding at equine beta-endorphin levels of 5000 nM. Radioligand degradation is discussed in Chapter 5.

Statistical Analyses

Data were analyzed by multiple analysis of variance and Pearson's correlation coefficients. Student's paired t-test was also performed where applicable. Significance was assigned at the $P<0.05$ level.

C. RESULTS

1. Physiologic Responses

The exercise tests, consisting of one interval training session, resulted in peak heart rates averaging 187.8 ± 14.6 beats/min (n=15). The peak heart rates during exercise were statistically elevated as compared to baseline levels ($P<0.001$), and reached near maximal levels (Figure 27). Lactate concentrations were also significantly increased from resting levels ($P<0.001$), and greatly exceeded anaerobic threshold levels of 4 mmol/L (Figure 28). The combination of near maximal heart rates and lactate concentrations exceeding anaerobic threshold suggest that the horses reached near maximal exercise intensity. Maximal exercise intensity, i.e. 100% VO_{2max}, is usually associated with heart rates of 220 beats/min or greater and lactate concentrations exceeding 4 mmol/L or anaerobic threshold [Persson, 1983]. Therefore, the peak heart rates and lactate concentrations reached following the exercise tests are consistent with values obtained in other studies [Persson, 1983] and suggest that the horses reached exercise intensity levels of approximately 85% VO_{2max}. Beta-endorphin concentrations are reported to significantly increase only after near-maximal or maximal exercise, i.e. 80% VO_{2max} or greater [Gambert, *et al.*, 1981; Goldfarb, *et al.*, 1987]. Beta-endorphin concentrations were also

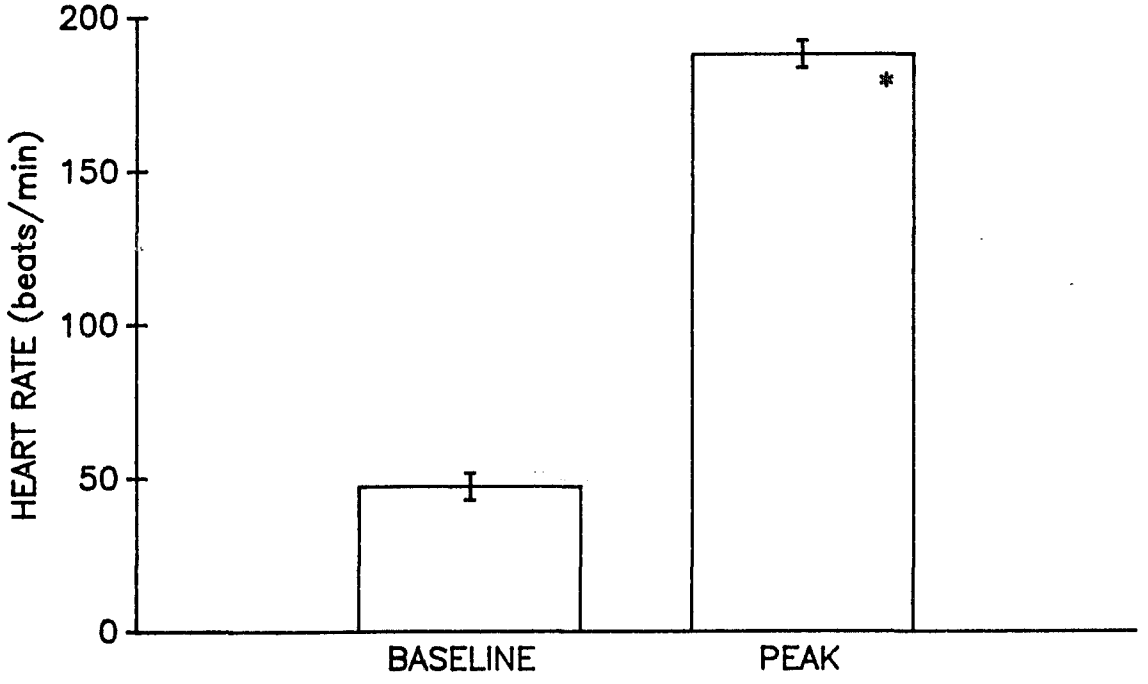

Figure 27. Heart rates in conditioned horses at rest (baseline) and peak heart rates during exercise. (*) indicates statistical difference from baseline ($P<0.001$).

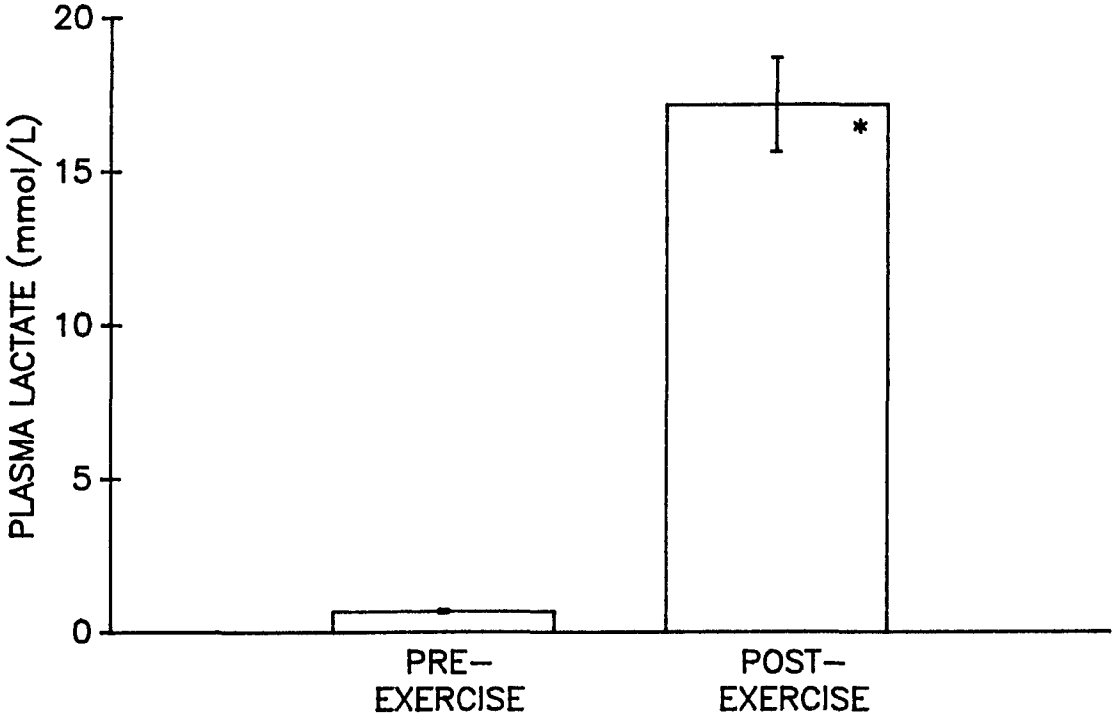

Figure 28. Lactate concentrations in conditioned horses immediately prior to exercise and post-exercise. (*) indicates statistical difference from pre-exercise values ($P<0.001$).

significantly increased following the exercise tests ($P<0.029$), therefore confirming that the horses reached a level of exercise of at least 85% VO_{2max} (Figure 29).

2. Binding Parameter Evaluation

Figure 30 depicts the cumulative Scatchard plots of all horses in each group and illustrates the overall differences between the groups. Each curve was calculated from lymphocytes from each horse, under each condition, and contained 6-8 points with 3 replicates each. Scatchard/Rosenthal plots of the binding data revealed that exercise resulted in an increased or steeper slope of the linear regression line in fit horses compared to values obtained prior to exercise. Sedentary horses at rest also had plots with steeper slopes than did fit horses at rest. The overall slopes and corresponding correlation coefficients are detailed in Table 12. An increase in or steeper slope results in a decrease in the dissociation constant, or K_d, and also results in a decrease in B_{max}, or total number of binding sites.

The K_d and B_{max} can be calculated from the y-intercept and the slope. Scatchard analysis of binding data from each horse was performed and the resultant binding parameters are detailed in Table 13. The dissociation constant for fit horses at rest ranged from 58 to 369 pM, while in sedentary horses the range was from 48 to 194 pM. The range in fit horses after exercise was from 38 to 162 pM. The average K_d values for each group are depicted graphically in Figure 31. The dissociation constant was significantly decreased by exercise in the fit horse, while sedentary horses had significantly lower K_d values than fit horses at rest ($P<0.02$). The results suggest that intense exercise acutely increases the affinity of the receptor for the ligand. It also suggests that the receptors in

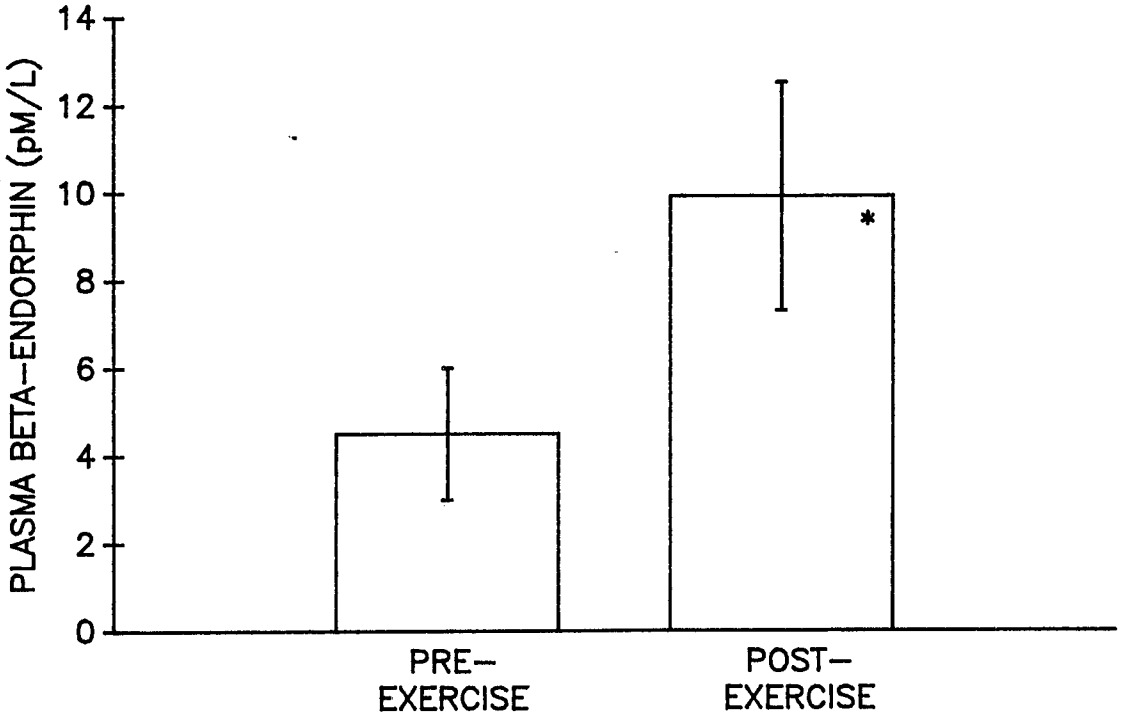

Figure 29. Beta-endorphin levels in conditioned horses immediately prior to and post exercise. (*) indicates statistical difference from pre-exercise values ($P<0.029$).

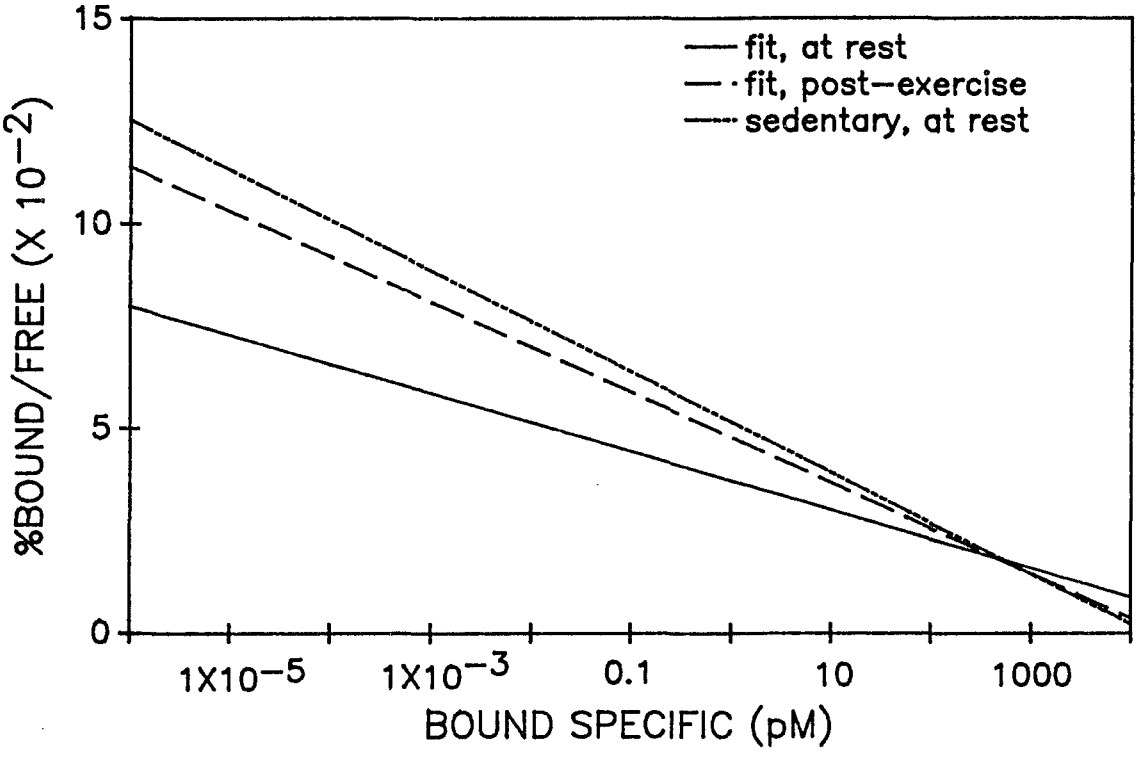

Figure 30. Scatchard analysis of beta-endorphin receptors on equine lymphocytes collected from fit horses at rest, fit horses after exercise and sedentary horses at rest.

TABLE 12. Cumulative Scatchard Plot Parameters

Group	Y-intercept*	Slope	Correlation Coefficient
Fit horses, at rest	3.7252×10^{-2}	-7.1140×10^{-3}	0.61474
Fit horses, post exercise	4.7780×10^{-2}	-1.1046×10^{-2}	0.50330
Sedentary horses, at rest	5.1583×10^{-2}	-1.2320×10^{-2}	0.67066

*Y-intercept values are calculated estimates.

TABLE 13. Effects of Exercise and Training on Beta-endorphin Receptor Binding

	Fit, at rest		Fit, after exercise			Sedentary, at rest	
Horse ID #	K_d*	B_{max}*	K_d	B_{max}	Horse ID #	K_d	B_{max}
106	189.76	0.8128	162.03	0.5441	74	67.47	0.3279
105	303.80	1.0405	149.50	0.9007	34	48.28	0.5083
135	162.90	0.5205	115.49	0.3053	93	65.26	0.5799
84	58.10	0.3238	106.90	0.5509	84	122.70	0.6166
107	160.78	0.5416	94.54	0.7473	107	100.14	0.5042
83	136.54	0.6819	37.97	0.0643	109	56.20	0.6183
104	112.49	0.5579	38.25	0.2271	95	89.38	0.3046
138	369.06	1.3050	125.80	0.4858	138	75.07	0.4808
56	60.60	0.3811	76.56	0.5708	136	72.82	0.5607
77	119.88	0.6442	52.51	0.2947	103	104.59	0.5914
57	236.89	1.0069	66.98	0.2238	57	194.45	1.5446
73	85.16	0.3569	70.65	0.2536	82	53.95	0.3215
82	106.95	1.0182	47.51	0.5166			
115	88.02	0.6517	86.39	0.5031			
108	113.90	0.4287	46.72	0.3928			
Mean	153.65	0.6848	85.19	0.4394		87.53	0.5799
SEM	22.99	0.0757	10.28	0.0565		11.69	0.0938

*K_d values reported in units of pM, B_{max} values reported in units of fmoles/10^6 cells.

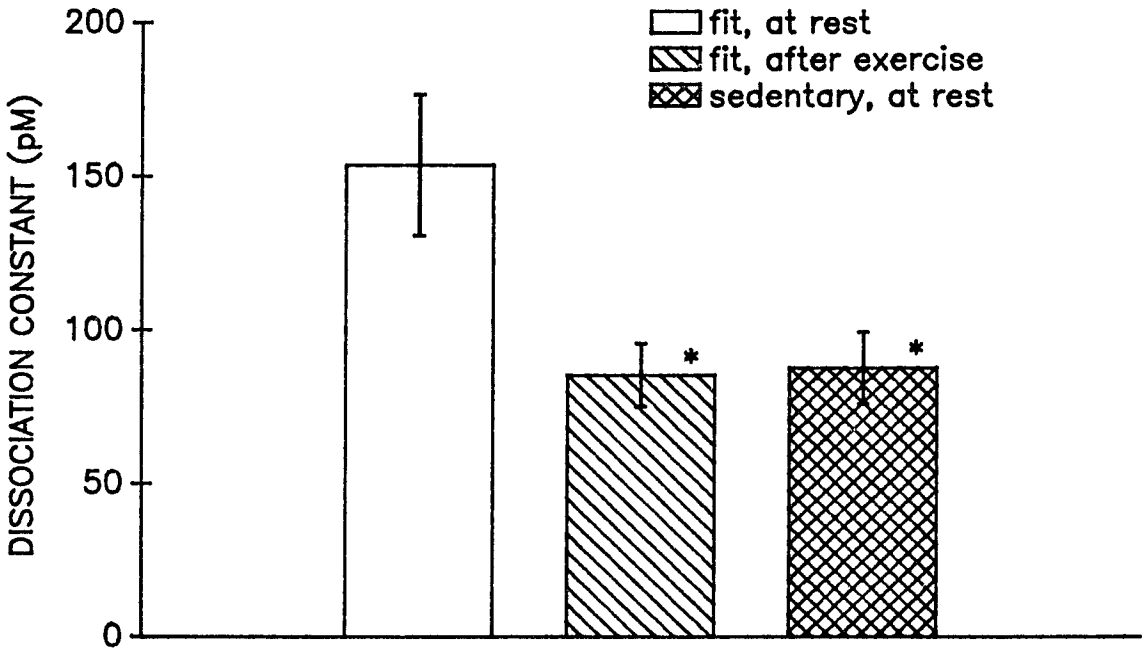

Figure 31. Dissociation constants (K_d) of beta-endorphin receptors on equine lymphocytes derived from Scatchard analysis of binding data. (*) indicates statistical difference from resting values in fit horses ($P<0.02$).

sedentary horses have higher affinity for the beta-endorphin than do receptors in fit horses. The total number of binding sites, i.e. B_{max}, also changed in response to exercise. The B_{max} values in fit horses at rest ranged from 0.32 to 1.30 fmoles/10^6 cells, while the values after exercise ranged from 0.06 to 0.90 fmoles/10^6 cells. The B_{max} values in sedentary horses ranged from 0.3 to 1.5 fmoles/10^6 cells. The average B_{max} values are depicted graphically in Figure 32. The total number of binding sites was significantly decreased after exercise ($P<0.01$) but did not change significantly with training. Although the mean B_{max} value was lower in sedentary horses than in fit horses, it was not significantly different. The range of B_{max} values showed no difference between fit and sedentary horses.

3. Correlations

Correlations were performed in order to determine if a relationship existed between physiologic indices of exercise and fitness, and binding parameters. In the fit horse, there was a positive correlation between beta-endorphin concentrations and lactate concentrations and heart rate (Table 14). There was also a strong positive correlation between heart rate and lactate concentrations in the fit horse. There was a negative correlation between heart rate and both K_d and B_{max} in the fit horse. In the fit horse after exercise, there was a strong positive correlation between lactate concentrations and K_d. These correlations indicate that there are parallel fluctuations of beta-endorphin levels, heart rates and lactate concentrations over all times. However, increased heart rates corresponded to decreased binding parameters over all times, while increased lactate concentrations corresponded to higher K_d's following exercise. In all horses, under all

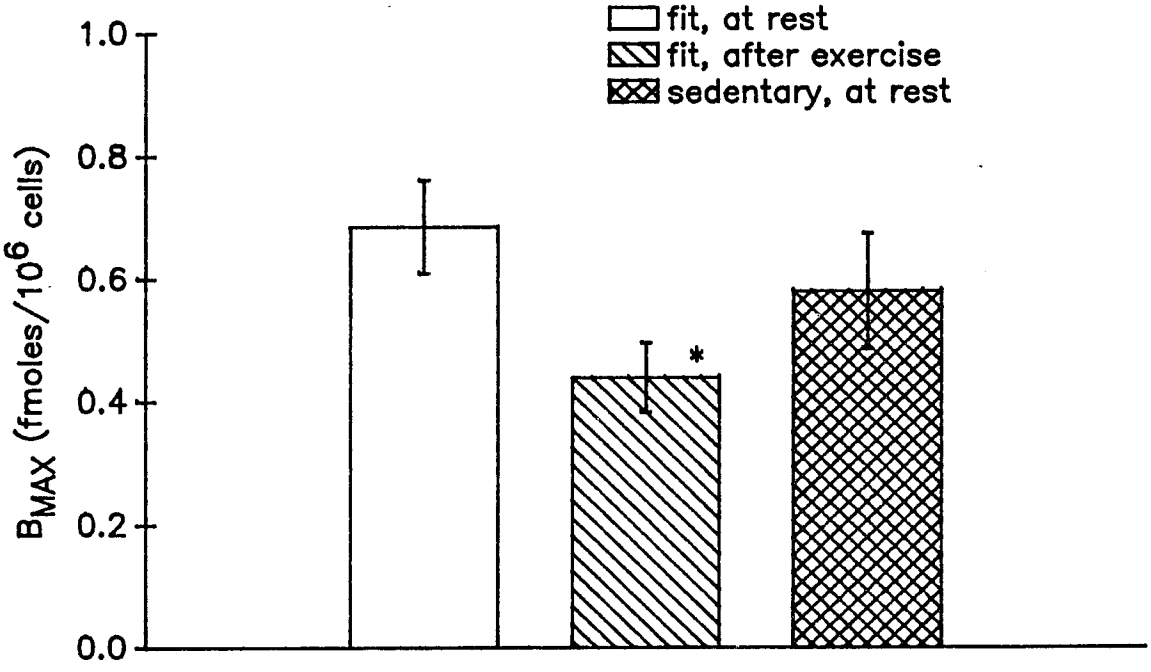

Figure 32. Total number of binding sites (B_{max}) of beta-endorphin lymphocyte receptors derived from Scatchard analysis of binding data. (*) indicates statistical difference from resting values in fit horses ($P<0.01$).

TABLE 14. Pearson's Correlation Coefficients for Exercise Test and Binding Data Parameters

Comparison	r_{obs}	Prob($r>[r_{obs}]/H_o$: p=0)*
Beta-endorphin vs. Lactate		
Fit horses, pre and post exercise	0.40280	0.0273
Beta-endorphin vs. Heart rate		
Fit horses, pre and post exercise	0.45520	0.0381
Lactate vs. Heart rate		
Fit horses, pre and post exercise	0.86025	0.0001
Heart rate vs. K_d		
Fit horses, pre and post exercise	-0.55401	0.0092
Heart rate vs. B_{max}		
Fit horses, pre and post exercise	-0.57330	0.0066
K_d vs. Lactate		
Fit horses, post exercise	0.78984	0.0005
K_d vs. B_{max}		
Fit horses, at rest	0.83293	0.0001
Fit horses, after exercise	0.63804	0.0105
Sedentary horses, at rest	0.82940	0.0008

*Probability estimate of acceptance of the null hypothesis.

conditions, there was a strong positive correlation between K_d and B_{max}.

D. DISCUSSION

The changes in lymphocyte beta-endorphin receptor binding parameters in response to exercise illustrate that beta-endorphin may be involved in modulating immune function following exercise. Enhanced affinity of the receptor following exercise suggests that beta-endorphin has increased ability to modulate immune function at this time. The dissociation constant was also lower in the sedentary population of horses as opposed to the fit horses. This again suggests that the higher affinity of the receptor for the ligand in the sedentary horses could result in increased ability of beta-endorphin to modulate immune function in these horses. Kurcz, *et al.* [1988] demonstrated suppression of the blastogenic response to mitogens in equine lymphocytes following exhaustive treadmill exercise. Increased beta-endorphin concentrations following exercise suggest that beta-endorphin may be involved in the suppression of immune function following exercise. Consequently, the question of the mechanism by which beta-endorphin alters immune function is raised. The existence of receptors on equine lymphocytes for beta-endorphin suggest that beta-endorphin modulates equine immune function through interaction with these receptors. If this is so, increased affinity for the receptor following exercise may result in augmented suppression of lymphocyte function. Beta-endorphin receptors in the sedentary horses had higher affinity than did receptors in the fit horses. This also suggests that stress, which would result in increased beta-endorphin levels in sedentary horses, could cause augmented suppression of lymphocyte function as compared to a fit

horse. Based on these results, it would appear that a sedentary horse has decreased immune function in response to stress when compared with a fit horse and that maximal exercise results in a transient suppression in immune function in the horse.

The total number of binding sites also decreased significantly following exercise in the fit horses. These results suggest that there is an upregulation of the receptor to one of higher affinity and a decrease in the total number of receptors. There was a strong positive correlation between the K_d and B_{max} values which confirms the relationship between the affinity of the receptor for the ligand and the number of receptors. As the affinity of the receptor increases, the number of receptors required to elicit the same response becomes smaller. Generally, low affinity receptors are more abundant than high affinity receptors. Therefore, a decrease in lower affinity receptors may explain the decrease in B_{max} noted following exercise. However, there is also the possibility of receptor internalization. Receptor internalization occurs via a high affinity binding site in murine thymoma cells [Schweigerer, *et al.*, 1985a]. It is therefore possible that an increase in high affinity binding sites, along with increased plasma beta-endorphin levels following exercise, leads to increased receptor-ligand interactions and therefore increased receptor-ligand complex internalization, and thus a decrease in number of available binding sites on equine lymphocytes. The results following exercise differed from those noted in the horses at rest. The number of binding sites in sedentary horses did not differ significantly from the number of binding sites in fit horses at rest, although the average B_{max} value was lower in sedentary horses. These results support the theory that the decreased number of binding sites following exercise result from receptor-ligand complex

internalization. If the decreased B_{max} following exercise resulted from receptor internalization, it would then most likely be a transient response to increased beta-endorphin levels and therefore would not be seen in resting horses.

Near maximal exercise intensity was reached by the fit horses during the exercise tests. The positive correlations found between beta-endorphin and lactate concentrations and heart rate, as well as the positive correlation between heart rate and lactate concentrations confirm that these parameters are good indices of exercise intensity. Interestingly, both K_d and B_{max} were negatively correlated with heart rate in fit horses. These correlations suggest that as exercise intensity increases there is a corresponding decrease in K_d and B_{max}, which results in an increase in receptor affinity and decrease in number of binding sites. Therefore, as exercise intensity increases or becomes more stressful, the affinity of the receptor for beta-endorphin increases, thereby augmenting the suppression of the immune response. Again, the corresponding decrease in binding sites may be due to a decrease in low affinity sites or an increase in high-affinity receptor-ligand complex internalization. However, changes in binding parameters following exercise may result from increases in body temperature or decreases in pH due to increased lactate levels.

In contrast, there was a positive correlation between K_d and lactate concentrations in fit horses after exercise. In other words, as lactate concentrations increased so did the K_d values. These results appear to conflict with the negative correlation between heart rate and K_d. It appears that the fit horses with the higher lactate concentrations after exercise also had lower affinity of the receptor for beta-endorphin following exercise. It

has been suggested that trained human athletes produce greater amounts of lactic acid in response to maximal exercise than do untrained individuals [Mougin, *et al.*, 1987]. Therefore, the horses which were in better physical condition may have released greater amounts of lactic acid, had lower heart rates and also had a corresponding lower affinity of the receptor for the ligand. This would again suggest that the fitter animals' response to exertional stress results in less immune suppression and therefore improved immune function.

The range of K_d values noted in this experiment were slightly higher than the K_d value reported in Chapter 5 (48-194 pM vs. 17 pM). The higher values reported in this study may be due to assay differences. The dissociation constant reported in Chapter 5 was calculated from a Scatchard analysis of a saturation isotherm, while the K_d values reported in this study were calculated from Scatchard analysis of competition-inhibition curves. Scatchard analysis of competitive displacement binding studies, rather than saturation isotherms, are commonly used due to cost and technical considerations. It is considered to be a valid use if the ligand-receptor interaction is simple competitive inhibition [Weiland and Molinoff, 1981]. However, artifactually nonlinear Scatchard plots will result if the radioligand and the unlabeled ligand bind with unequal affinity [Taylor, 1975]. Also, with all Scatchard plots, there is the problem of the inappropriate definition of nonspecific binding, which will also result in a nonlinear Scatchard plot [Limbird, 1986]. In this study, the Scatchard plots of the competition binding data resulted in a nonlinear Scatchard plot (concave upward) if the X-axis was on a linear scale. This type of plot may result from multiple orders of noninteracting binding sites, the existence of

multiple affinity states of the receptor, or negative cooperativity [Limbird, 1986]. The nonlinear Scatchard plots parallel the results obtained from the competition-inhibition curves performed in the previous study [Chapter 5]. In both cases, the shape of the curves suggest that there is a more complex ligand-receptor interaction occurring than when a single ligand interacts with a single population of receptors.

In order to obtain one overall K_d and B_{max} value from Scatchard plots, the plot was linearized by performing a logarithmic transformation of the data. Many investigators subjectively split nonlinear concave upward plots into two sections, representing a high affinity and low affinity portion, upon which linear regression analysis is performed. However, this is a totally subjective procedure, which introduces a large amount of error, as it reduces the number of points used to calculate the binding parameters. Therefore, in an effort to reduce the error and increase the ability to detect a difference between treatment groups, the plot was linearized resulting in an "overall" K_d and B_{max} values. The use of this technique may explain the difference in K_d values obtained in this study as compared with the previous study. Also, when performing Scatchard analysis of competitive displacement studies, the amount of free ligand reaches very high, nonphysiological levels, as compared the ligand levels in a saturation isotherm. This may result in an artifactually low affinity site which has no physiological function. Although the "overall" K_d and B_{max} values obtained in this study may not be quantitative, they can be accurately used to detect a difference between treatment groups. Scatchard plots, in general, give only estimates of the true dissociation constant due to inherent problems which lead to magnification of error. However, they can be legitimately used to evaluate

changes as a result of a "treatment" as opposed to a "control" [Munson and Rodbard, 1983].

In conclusion, exercise and physical conditioning alter the binding parameters of beta-endorphin receptors on equine lymphocytes. Intense exercise increases the affinity of the receptor for beta-endorphin while physical conditioning decreases the affinity of the receptor for beta-endorphin. If beta-endorphin suppresses lymphocyte function, as intense exhaustive exercise has been reported to do, then intense exercise or exertional stress may exacerbate immune function while physical conditioning may improve the immune response to stress through the interaction of beta-endorphin with its receptors on equine lymphocytes.

CHAPTER SEVEN

MODULATION OF MITOGEN-INDUCED EQUINE LYMPHOCYTE PROLIFERATIVE RESPONSES BY BETA-ENDORPHIN

A. INTRODUCTION

The identification of opioid receptors on human and murine lymphocytes has generated considerable interest in evaluating the immunoregulatory actions of endogenous opioid peptides [Mehrishi and Mills, 1983; Carr, *et al.*, 1989; Sibinga and Goldstein, 1988]. Beta-endorphin is coreleased from the pituitary with adrenocorticotropin in response to a variety of stressors, such as exercise, surgery and noxious or painful stimuli, and therefore represents a possible mediator of neural regulation of immune function [Cohen, *et al.*, 1986; Guillemin, *et al.*, 1977]. Current studies demonstrate modulation of a number of immune cell functions, such as lymphocyte proliferative responses and natural killer cell activity, by beta-endorphin [Sibinga and Goldstein, 1988]. Furthermore, the finding that beta-endorphin and related proopiomelanocortin derived peptides are produced by activated lymphocytes suggests that there exists a complete modulatory loop between the immune and neuroendocrine systems [Morley, *et al.*, 1987].

The effect of endogenous opioid peptides on lymphocytes has commonly been assessed by testing the influence of these peptides on mitogen-induced lymphocyte proliferation *in vitro* [Murgo, *et al.*, 1986]. The mitogens most commonly used are Concanavalin A (ConA), a T and B cell mitogen, phytohemagglutinin (PHA), a T cell mitogen, and Pokeweed mitogen (PWM), a B cell mitogen which requires the presence

of T helper cells. Enhancement of ConA and PHA induced proliferation in rat splenocytes by beta-endorphin has been reported [Gilman, *et al.*,1982; Kusnecov, *et al.*, 1989]. Beta-endorphin also enhances murine ConA-induced lymphocyte proliferation [Gilmore and Weiner, 1989]. In these studies, the modulation of lymphocyte proliferative responses by beta-endorphin was not reversed by naloxone or methionine-enkephalin and appeared to require the carboxy terminal portion of the molecule for activity. Therefore, it was concluded that beta-endorphin influences lymphocyte proliferation through interaction with a nonopioid receptor.

Reports of beta-endorphin modulation of human lymphocyte proliferation generally indicate suppression of the mitogenic response [McCain, *et al.*, 1982; Puppo, *et al.*, 1985]. However, there are recent reports that beta-endorphin is capable of both enhancing and suppressing the proliferative response of human lymphocytes to the mitogens ConA and PHA [Heijnen, *et al.*, 1987; McCain, *et al.*, 1987]. These studies suggested that the effect was dependent upon both peptide concentration and donor, and that the activity of the peptide resided in the carboxy terminal sequence. These studies indicate that beta-endorphin is capable of modulating the proliferative response of lymphocytes, perhaps through interaction with a nonopioid receptor, and that the effect may be dependent on the mitogen used, dose, individual and species.

Recent work in our laboratory has demonstrated receptors for beta-endorphin on equine lymphocytes [Chapter 5]. These receptors appear to be opioid receptors of the mu or delta class and their affinity for the ligand, beta-endorphin, is altered by both exercise and physical conditioning [Chapter 6]. As yet, the ability of beta-endorphin to modulate

equine lymphocyte function has not been studied. Therefore, in order to clarify the role beta-endorphin receptors play in equine immune function, equine lymphocyte proliferative responses to beta-endorphin were assessed using the mitogens Concanavalin A, phytohemagglutinin and pokeweed mitogen.

B. MATERIALS AND METHODS

Lymphocyte collection and preparation

Blood was collected from the jugular vein of healthy, conditioned Thoroughbred horses into 20 ml evacuated siliconized glass tubes containing heparin at a concentration of 10 U/ml as an anticoagulant. Two experiments were performed, one using optimal mitogen concentrations and a later experiment using suboptimal mitogen concentrations. In the first experiment eight Thoroughbred horses, 5 female and 3 male with a mean age of 5.9±1.7 years, were used. In the second experiment, six Thoroughbred horses, all female in order to control assay variability, with a mean age of 5.5±1.4 years were used. Heparinized tubes were prepared by injecting 100 U of preservative free heparin dissolved in Hanks' Balanced Salt Solution into 20 ml evacuated tubes. The heparin solution was sterilized prior to use by filtering through 0.22 micron filters[1] into sterile 50 ml centrifuge tubes[2]. Two 20 ml tubes were collected from each horse.

The whole blood was centrifuged at 400 x g for 10 minutes at room temperature.

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[1]Acrodisc syringe filters, 0.2 micron, Gelman Sciences, Ann Arbor, Mich.

[2]Corning 50 ml polypropylene centrifuge tubes with cap, sterile, Corning Glass Works, Corning, N.Y.

Using sterile techniques, the plasma was removed and discarded and the buffy coat transferred to a sterile 15 ml conical centrifuge tubes[3]. The buffy coat from each tube was diluted up to a total volume of 10 ml in calcium and magnesium free Hanks' Balanced Salt solution (CMF Hanks). The cell suspension was mixed well and layered over 4 ml of room temperature Ficoll. The cell suspension was then centrifuged at 600 x g for 30 minutes at room temperature. The upper supernatant layer was removed and discarded and the interface, which contained the mononuclear cells, was removed and placed in a sterile 15 ml conical centrifuge tube containing 8 ml of CMF Hanks. The cell suspension was mixed well and centrifuged at 400 x g for 10-15 minutes. The supernatant was then decanted, pellet disrupted, and 10 ml of fresh CMF Hanks' was added. The cell suspension was centrifuged at 350 x g for 10 minutes and the supernatant was decanted. The cells were then resuspended in 5 ml of assay medium, RPMI 1640[4] supplemented with 10% FBS[5], 100 U/ml penicillin[6], 100 µg/ml streptomycin[7] and 10^{-5}M 2-mercaptoethanol[8]. Lymphocyte concentration was determined by counting on a hemacytometer. Cell concentration was then adjusted to a final concentration of 1 X 10^6

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[3]Corning 15 ml polypropylene centrifuge tube with cap, sterile, Corning Glass Works, Corning, N.Y.

[4]RPMI 1640 with L-glutamine, without sodium bicarbonate, Sigma Chemical Co., St. Louis, Mo.

[5]Fetal bovine serum, Gibco Laboratories, Grand Island, N.Y.

[6]Penicillin G potassium for injection, E.R. Squibb and Sons, Princeton, N.J.

[7]Streptomycin sulfate, Pfizer, Inc., Roerig Division, New York, N.Y.

[8]2-mercaptoethanol, Aldrich Chemical Co., Milwaukee, Wi.

cells/ml using the assay media. Cell viability was assessed by trypan blue dye exclusion and always exceeded 90% viable cells.

Lymphocyte proliferation assays

The lymphocyte proliferation assays were performed in 96 well round bottom multi-well microtiter plates.[9] Cells were dispensed into the 96-well plates at a final concentration of 1 X 10^5 cells/well (100 µl of cell suspension at 1 X 10^6 cells/ml). Optimal mitogen concentrations were determined empirically. Equine lymphocytes (1 x 10^5 cells/well) were incubated with increasing concentrations of each mitogen in a total volume of 200 µl. Mitogen concentrations were: PHA[10] and PWM[11] at 0.1, 0.2, 0.4, and 0.8 µg/well, and ConA[12] at 0.25, 0.5, 1.0, and 2.0 µg/well. Cells were also incubated without mitogen for determination of basal activity. The cultures were set up in replicates of four and incubated at 39°C in a humidified atmosphere of air and 5% CO_2. After 48 hours, each well was pulsed with 1 µCi/5 µl of tritiated thymidine[13] and incubated for an additional 16-18 hours. The lymphocytes were then harvested onto glass fiber filter paper[14] using a cell harvester.[15] The paper discs were put in scintillation

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[9]Corning 96 well round bottom polystyrene cell wells, Corning Glass Works, Corning, N.Y.

[10]Phytohemagglutinin, M form, Gibco Laboratories, Grand Island, N.Y.

[11]Phytolacca Americana (Pokeweed), Sigma Chemical Co., St. Louis, Mo.

[12]Concanavalin A, Type IV-S, Sigma Chemical Co., St. Louis, Mo.

[13]Thymidine 5' triphosphate, tetrasodium salt [Methyl ^{3}H]-, Dupont Co., Wilmington, DE.

[14]Filter paper for Titertek cell harvester, Skatron, Inc., Lier, Norway.

[15]Automatic cell harvester, Model 7109, Skatron, Inc., Lier, Norway.

vials[16] and 1.5 ml of scintillation cocktail[17] was added. The newly synthesized ^{3}H-DNA, an indicator of blastogenesis, was quantified on a beta-scintillation counter.[18] Results were expressed as counts per minute (CPM).

In the first experiment, cells from each horse were incubated with optimal concentrations of mitogens: PHA and PWM at a final concentration of 2 μg/ml, and ConA at final concentration of 5 μg/ml. Cells were incubated with the mitogens alone and with mitogens and beta-endorphin at final concentrations of 10^{-11}, 10^{-9}, and 10^{-7} M. Lymphocytes were also incubated with beta-endorphin (10^{-7} M) and naloxone (10^{-5} M) with the mitogens. Cells from each horse were also incubated with beta-endorphin and beta-endorphin plus naloxone at all concentrations without the mitogens. Each well contained 1 x 10^{5} lymphocytes and appropriate concentrations of mitogens, beta-endorphin or naloxone in a final volume of 200 μl and all dilutions were made in assay medium. The cultures were set up in triplicate and then incubated at 39°C in a humidified atmosphere of air and 5% CO_2. After 48 hours, each well was pulsed with 1 μCi/5 μl of tritiated thymidine and incubated for an additional 16-18 hours. Cell were harvested and results analyzed as described previously.

The second experiment used the same procedure as the first with the exception of mitogen concentrations and the use of naloxone alone as well as in combination with beta-endorphin. In the second experiment, suboptimal mitogen concentrations were used:

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[16]Packard Picopro 4 ml scintillation vials, Packard Instrument Co., Downer Grove, Il.

[17]Ultima Gold liquid scintillation cocktail, Packard Instrument Co., Downer Grove, Il.

[18]Beckman beta-scintillation counter, Model LS 5000 TD, Beckman Instruments, Palo Alto, Calif.

PHA and PWM at final concentrations of 1 μg/ml and ConA at a final concentration of 2.5 μg/ml. In addition, the effects of naloxone were analyzed. Lymphocytes from each horse were also incubated with naloxone alone, mitogen and naloxone, and mitogen, naloxone and beta-endorphin at 10^{-7} M. Naloxone was used at a concentration of 10^{-5} M.

Statistical Analyses

Data were analyzed by Student's paired t-test. Significance was assigned at the $P<0.05$ level.

C. RESULTS

1. Determination of optimal mitogen concentrations

Optimal mitogen concentrations were determined by the concentration of mitogen which produced the highest level of lymphocyte proliferation as indicated by uptake of tritiated thymidine as an indicator of newly synthesized DNA. The results are depicted in Table 15. The greatest response to the mitogen PHA was seen at 0.8 μg/well (4 μg/ml). The highest level of proliferation in response to the mitogen PWM was noted at a concentration of 0.4 μg/well (2 μg/ml). ConA stimulated lymphocyte proliferation optimally at 1.0 μg/well (5 μg/ml). Both ConA and PWM exhibited a "prozone" effect, that is, inhibition of proliferation at high concentrations of mitogen. Although this effect was not observed with PHA in the concentration range of 0.1-0.8 μg/well, the next lower concentration of mitogen (2 μg/ml or 0.4 μg/well) from the optimal dose was chosen for the next experiment to ensure adequate cell stimulation.

TABLE 15. Lymphocyte Proliferative Responses to Increasing Mitogen Concentrations

Mitogen		Mean CPM ± SEM*
PHA		
	0.1 μg/well	21586 ± 1234
	0.2 μg/well	30237 ± 2141
	0.4 μg/well	43377 ± 2183
	0.8 μg/well	54234 ± 2351
ConA		
	0.25 μg/well	10701 ± 1290
	0.5 μg/well	28048 ± 1045
	1.0 μg/well	52234 ± 359
	2.0 μg/well	25983 ± 3071
PWM		
	0.1 μg/well	32932 ± 361
	0.2 μg/well	38326 ± 2001
	0.4 μg/well	42401 ± 1143
	0.8 μg/well	38679 ± 2698
Control		2197 ± 266

*Mean ± SEM of 6 horses.

2. Experiment 1: Optimal mitogen concentrations

In the first experiment, the effects of beta-endorphin on mitogen-induced lymphocyte proliferation were assessed using optimal concentrations of mitogen determined previously. Beta-endorphin at all concentrations, and a combination of beta-endorphin plus naloxone, had no effect on lymphocyte proliferation in the absence of mitogen (Figure 33). Beta-endorphin also had no significant effect on PHA induced lymphocyte proliferation at any concentration (Figure 34), however, it significantly enhanced both PWM and ConA stimulated lymphocyte proliferation at the lowest dose, 10^{-11} M, when compared to mitogen alone ($P<0.05$) (Figure 34). Naloxone did not alter the response to the mitogen PHA in the presence of beta-endorphin (Figure 35). Naloxone also did not alter the response to the mitogen PWM in the presence of beta-endorphin (Figure 36). However, naloxone at 10^{-5} M in combination with beta-endorphin at 10^{-7} M significantly enhanced the proliferative response to ConA ($P<0.05$) even though beta-endorphin at this dose had no significant effect (Figure 37). The effect of beta-endorphin at 10^{-7} M did not significantly differ from the effect of beta-endorphin plus naloxone on the ConA induced proliferation. The enhancement of the lymphocyte proliferative response suggests that beta-endorphin is capable of modulating equine immune function. The ability of naloxone to also enhance ConA induced proliferative responses suggests that naloxone possesses agonist properties in contrast to the current theory that it is a strict antagonist.

Due to the large individual variability and the small amount of significant effect found in the first experiment, another experiment was performed using suboptimal

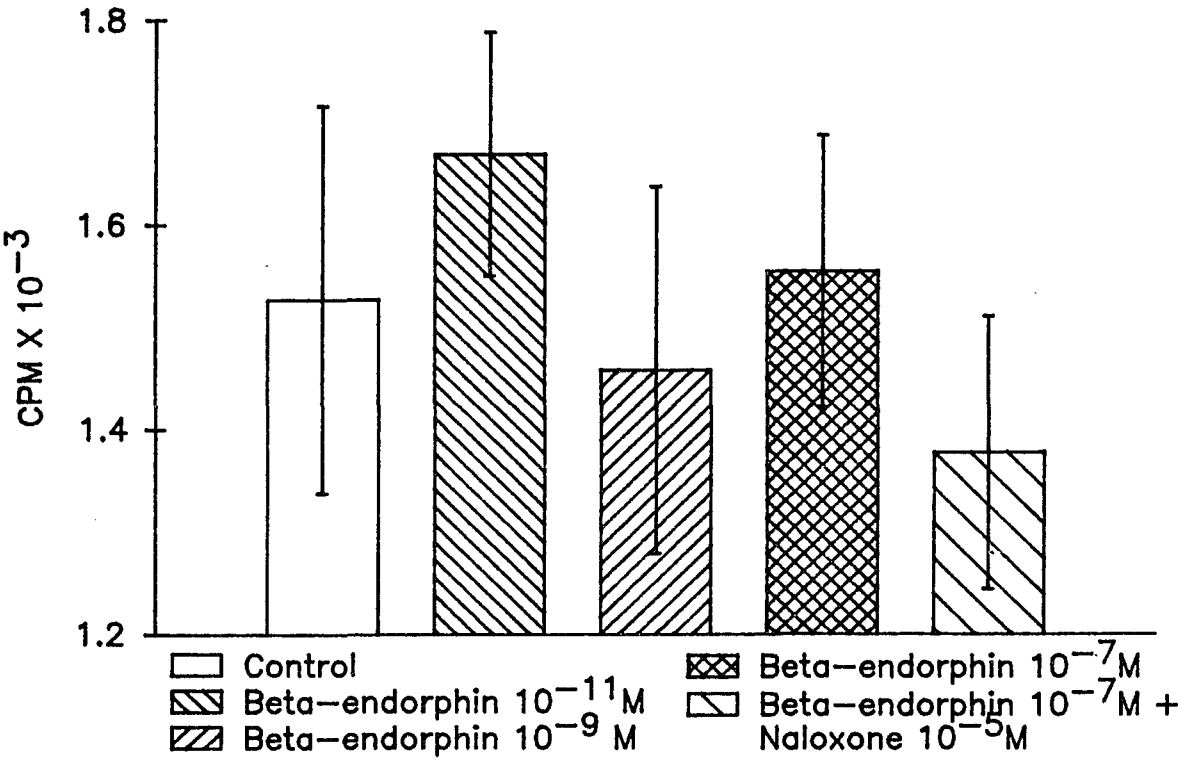

Figure 33. Effect of beta-endorphin and naloxone on basal proliferative activity of equine lymphocytes in experiment one (Mean±SEM of 8 horses).

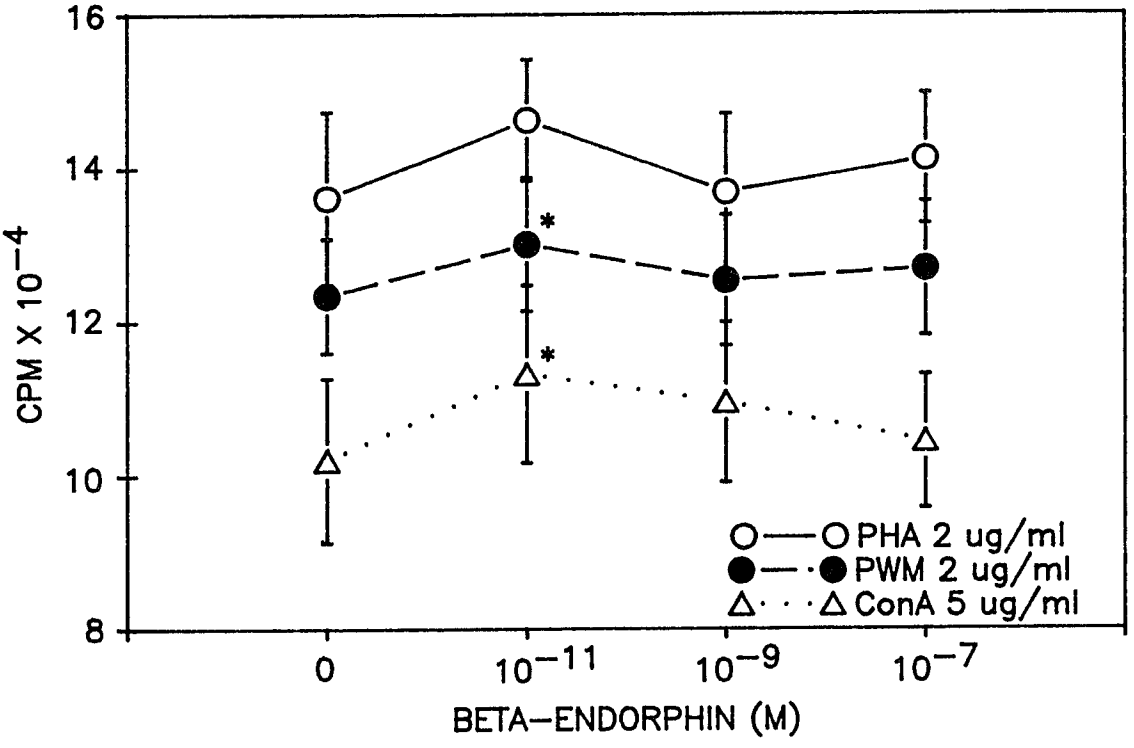

Figure 34. Effect of beta-endorphin on mitogen induced equine lymphocyte proliferation at optimal mitogen concentrations (Mean±SEM of 8 horses). (*) indicates statistical difference from mitogen control ([BE]=0) ($P<0.05$).

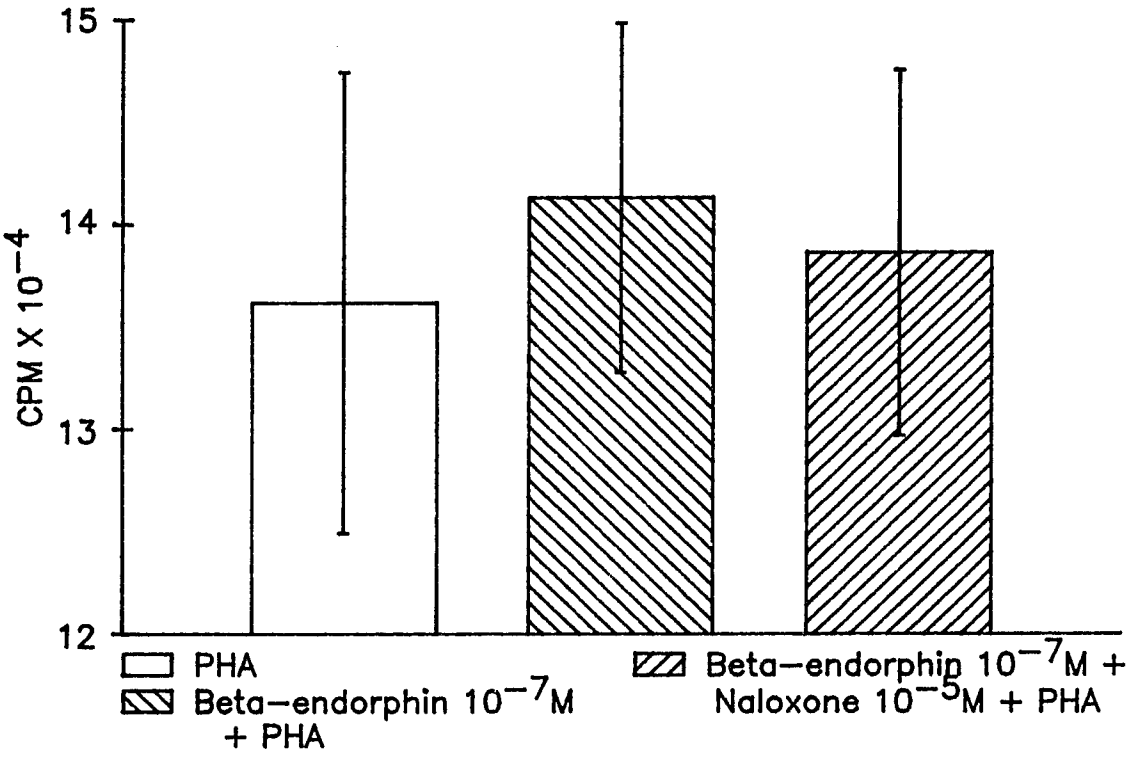

Figure 35. Effect of naloxone on beta-endorphin modulation of PHA-induced lymphocyte proliferation; PHA at 2 μg/ml (Mean±SEM of 8 horses).

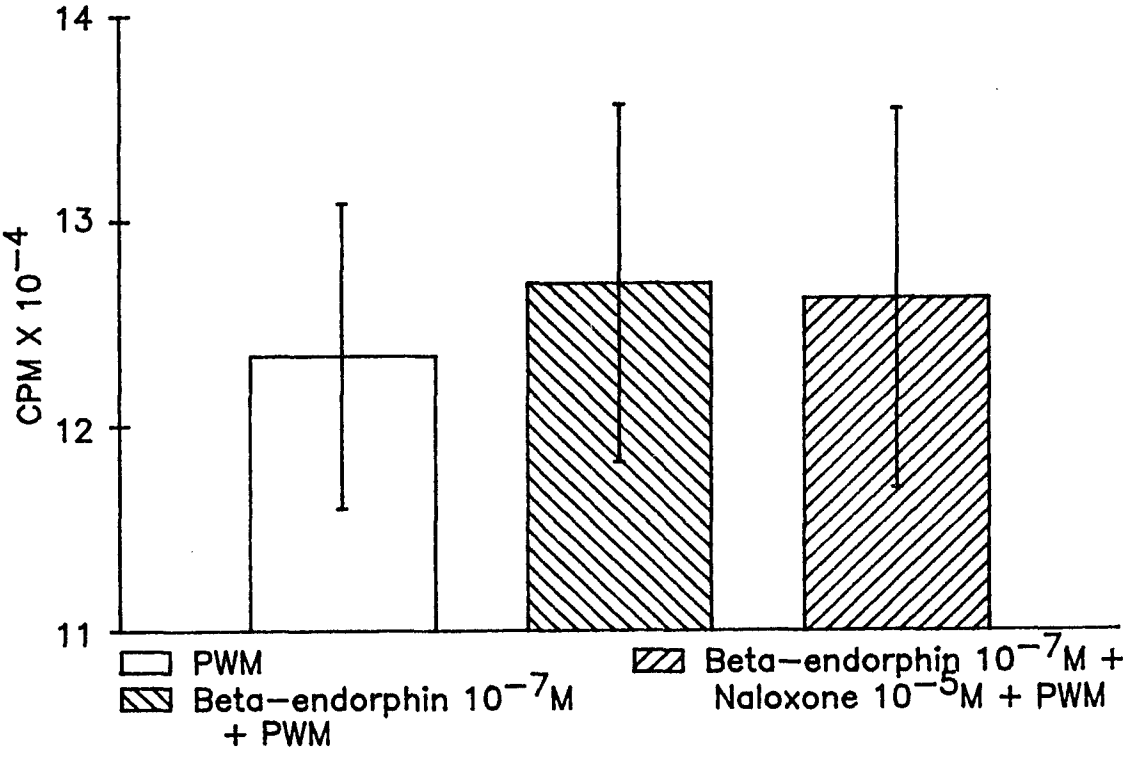

Figure 36. Effect of naloxone on beta-endorphin modulation of PWM-induced lymphocyte proliferation; PWM at 2 μg/ml (Mean±SEM of 8 horses).

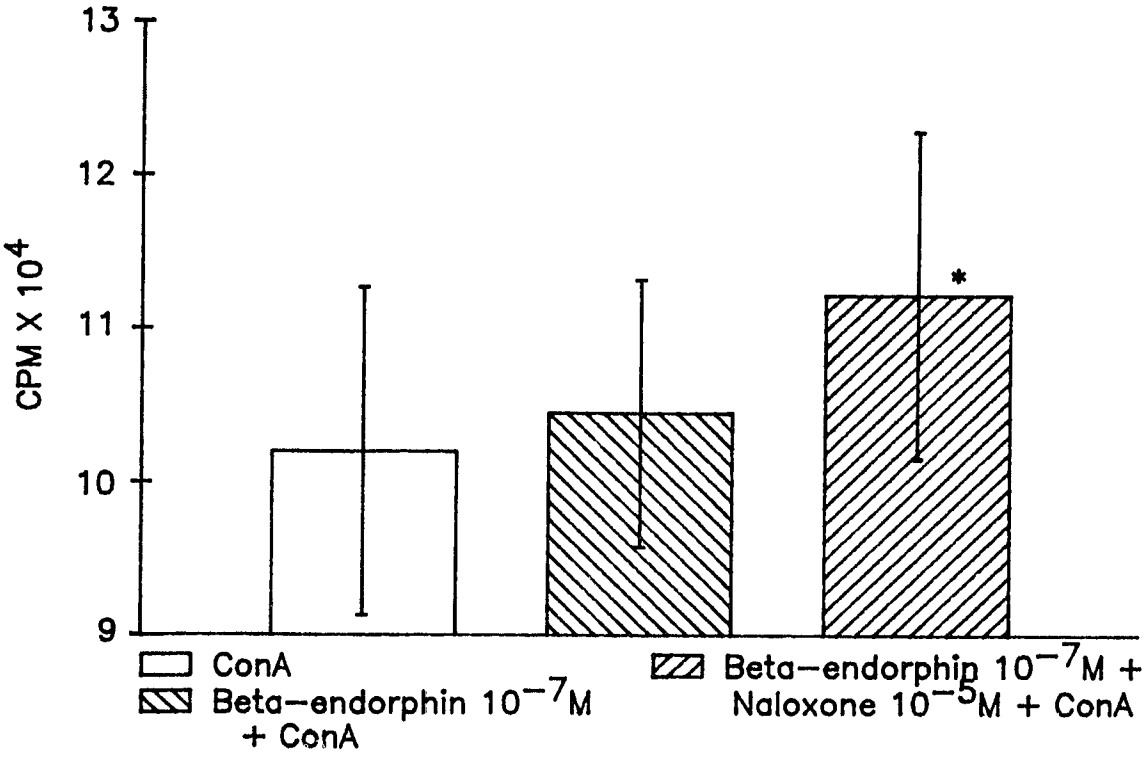

Figure 37. Effect of naloxone on beta-endorphin modulation of ConA-induced lymphocyte proliferation; ConA at 5 μg/ml (Mean±SEM of 8 horses). (*) indicates statistical difference from stimulation with ConA alone ($P<0.05$).

concentrations of mitogen. Optimal mitogen concentrations may have reduced the ability to detect an effect as the cells are maximally stimulated, thus suggesting that perhaps suboptimal mitogen concentrations would enhance detection of enhancement or suppression of the proliferative response. Horses of all one sex (female) were also used in the second experiment in order to reduce individual variability.

3. Experiment 2: Suboptimal mitogen concentrations

In the second experiment, beta-endorphin at all concentrations, naloxone, or beta-endorphin plus naloxone had no effect on lymphocyte proliferation in the absence of mitogens (Figure 38). Interestingly, beta-endorphin significantly suppressed the proliferative response to all the mitogens when used at suboptimal doses (Figure 39). Beta-endorphin suppressed the response to the mitogens PHA and PWM at all concentrations but suppressed ConA-induced lymphocyte proliferation only at the two higher doses. The addition of naloxone revealed interesting results. Naloxone also significantly suppressed PHA-induced proliferation as did the combination of beta-endorphin (10^{-7}M) and naloxone (10^{-5}M) ($P<0.01$) (Figure 40). There was no significant difference between the effect of naloxone alone and beta-endorphin plus naloxone on PHA-induced proliferative responses. Naloxone also had the same effect on PWM- and ConA-induced lymphocyte proliferation (Figures 41 and 42). In contrast to the first experiment, these results suggest that beta-endorphin does not enhance but rather suppresses mitogen induced proliferative responses, and therefore may suppress immune function in the horse.

Due to the conflicting results obtained with the two experiments, the results of the

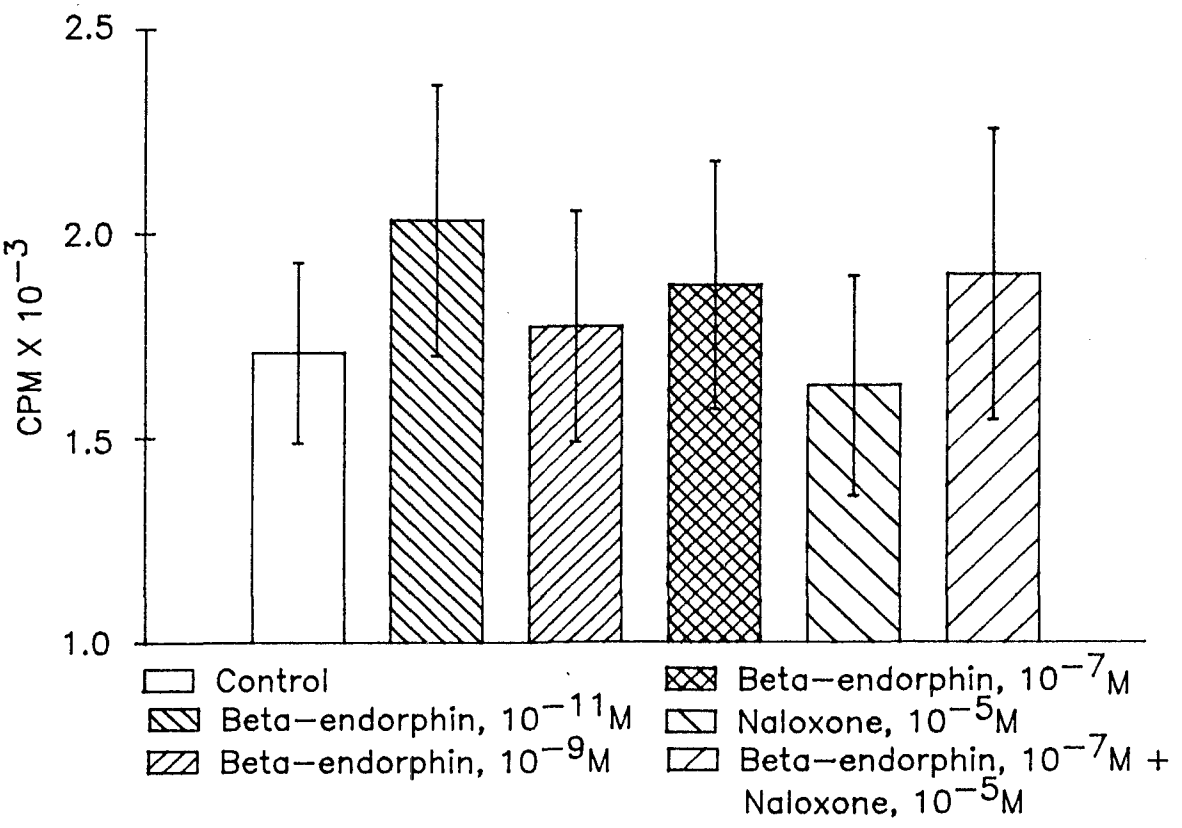

Figure 38. Effects of beta-endorphin and naloxone on basal proliferative activity of equine lymphocytes in experiment two (Mean±SEM of 6 horses).

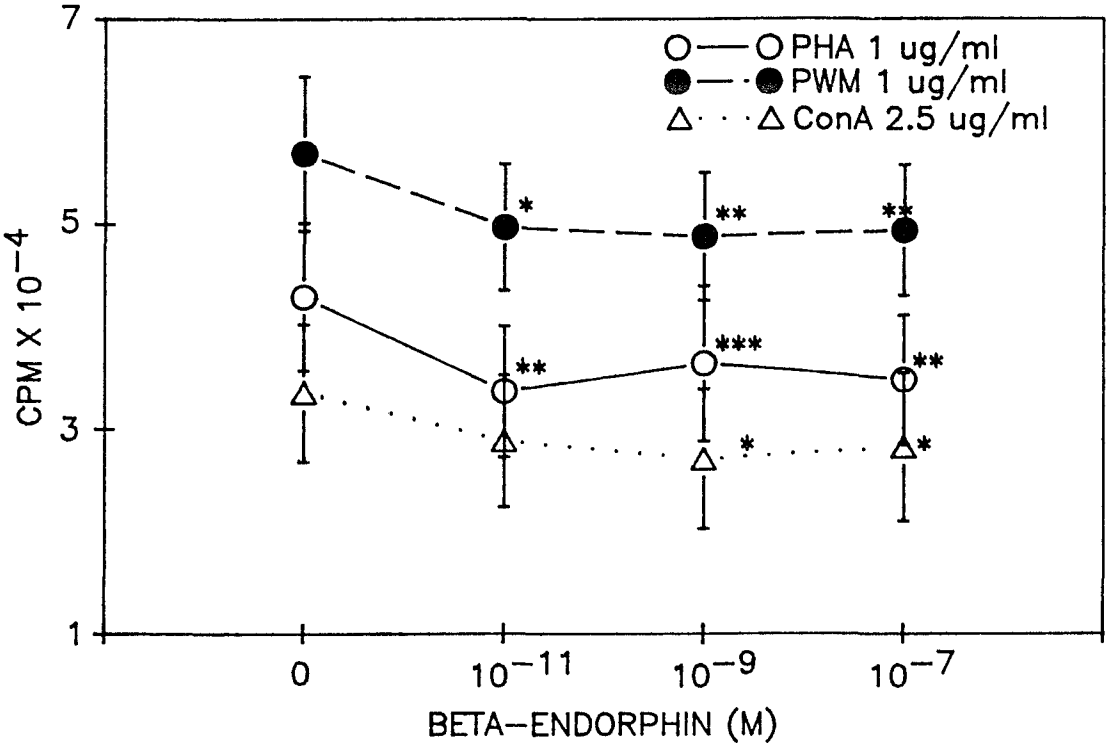

Figure 39. Beta-endorphin effects on mitogen induced lymphocyte proliferation at suboptimal mitogen concentrations (Mean±SEM, n=6). (*) indicates statistical difference from mitogen control ([BE]=0) (* P<0.05, ** P<0.01, *** P<0.001).

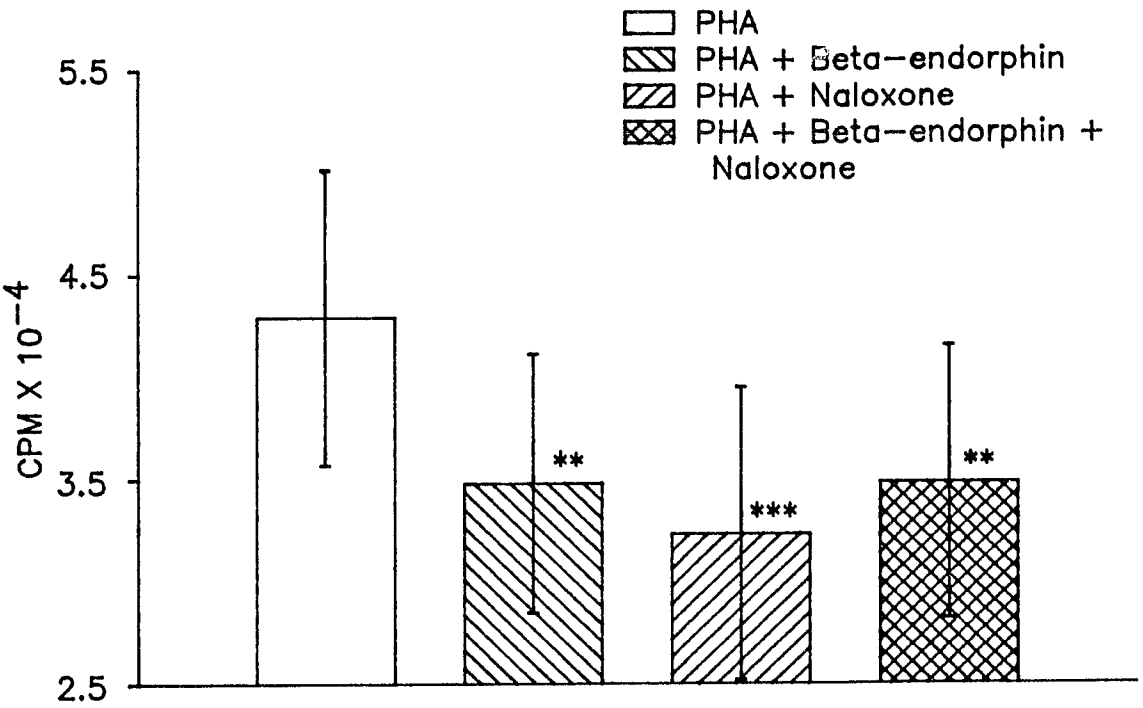

Figure 40. Effect of naloxone on PHA-induced lymphocyte proliferation with and without beta-endorphin; PHA at 1 μg/ml (Mean±SEM, n=6). (*) indicates statistical difference from mitogen control (*P<0.05, **P<0.01, ***P<0.001).

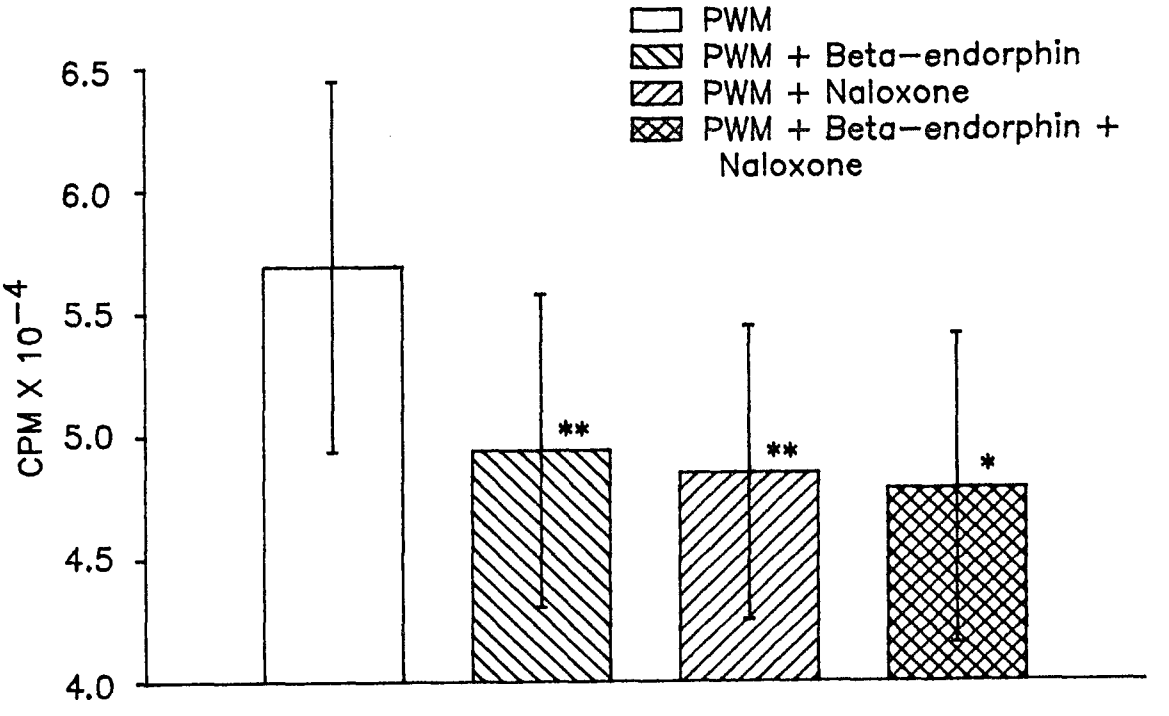

Figure 41. Effect of naloxone on PWM-induced lymphocyte proliferation with and without beta-endorphin; PWM at 1 μg/ml (Mean±SEM, n=6). (*) indicates statistical difference from mitogen control (*P<0.05, **P<0.01).

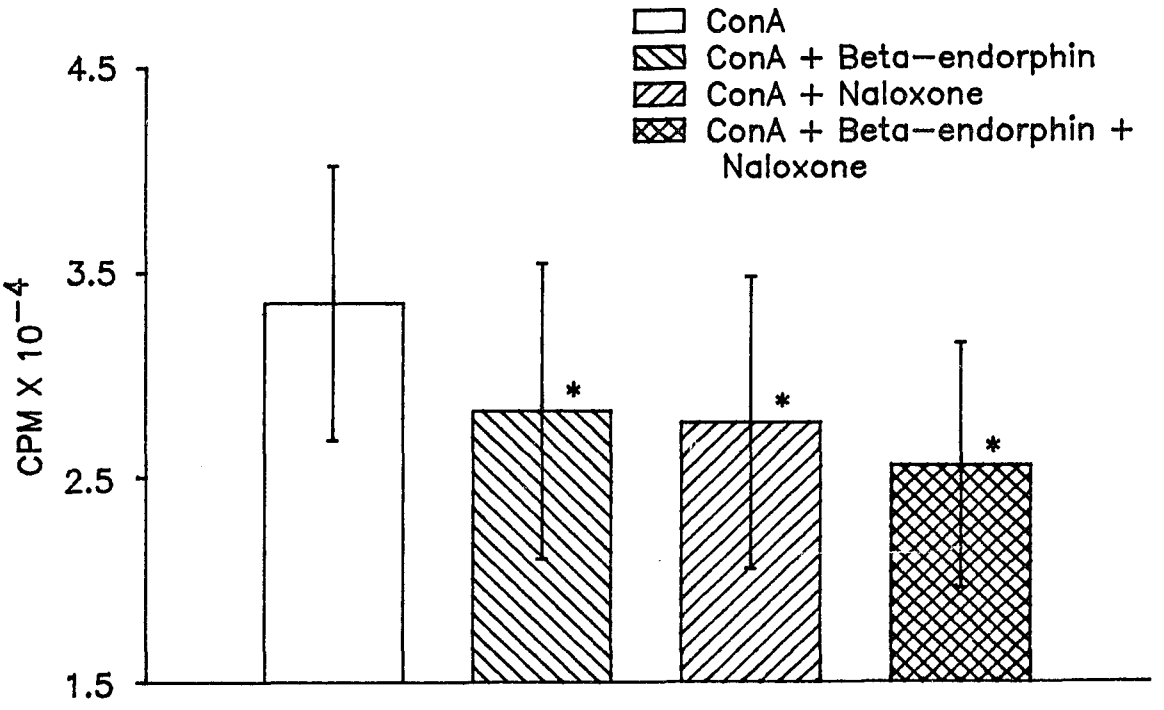

Figure 42. Effect of naloxone on ConA-induced lymphocyte proliferation with and without beta-endorphin; ConA at 2.5 μg/ml (Mean±SEM, n=6). (*) indicates statistical difference from mitogen control ($P<0.05$).

first experiment were analyzed according to sex of the horses in order to rule out sex as a contributor to the conflicting results. There was no significant difference between the responses of the male and female horses. It must be noted that the first experiment had a large amount of variability between individuals which was not present in the second experiment. It may be that the slight enhancement of the proliferative response in the first experiment was actually an artifact since it was not seen in the second experiment at the same beta-endorphin concentrations.

D. DISCUSSION

Endogenous opioid peptides may play a role in mediating stress induced behavioral and humoral responses [Morley and Levine, 1980]. Beta-endorphin is released from the pituitary in response to variety of stressors and has been implicated as a possible immunomodulatory agent [Cohen, *et al.*, 1986; Sibinga and Goldstein, 1988]. The results of this study clearly indicate that beta-endorphin suppresses equine lymphocyte proliferation in response to suboptimal mitogen concentrations. Although the modulatory action of beta-endorphin is negative at suboptimal mitogen concentrations, it appears to have a slight stimulatory effect at optimal mitogen doses. The stimulatory effects of beta-endorphin occurred only at the lowest doses (10^{-11} M), while the suppressive effects occurred primarily at the higher doses, suggesting that perhaps beta-endorphin exerts a biphasic response which is dependent upon the level of blastogenic response. This concept is supported by earlier work which demonstrated biphasic responses of human lymphocytes to glycyl-L-glutamine, the carboxy terminal dipeptide of beta-endorphin,

beta-endorphin and ACTH [Heijnen, *et al.*, 1987; McCain, *et al.*, 1987]. Although there may be a biphasic response to beta-endorphin, it is also possible that beta-endorphin is not capable of suppressing a maximal or optimal blastogenic response. Also, optimal mitogen concentration may not be physiologically relevant. Stimulation of lymphocytes by antigens *in vivo* most likely occurs at suboptimal levels, and therefore suboptimal mitogen concentrations may be more indicative of true *in vivo* activity.

The response to beta-endorphin appeared to be stimulation-dependent in that there was no suppression or enhancement of proliferative responses in the absence of mitogens, i.e. during basal activity. At optimal mitogen concentrations, beta-endorphin did not have any effect on the proliferative response to PHA, but enhanced the response to PWM and ConA at the lowest dose. Phytohemagglutinin stimulates the blastogenic response in T cells, while ConA stimulates both T and B cells, and PWM stimulates B cells although it requires T helper cells. Therefore, at optimal mitogen concentrations, it appears as though beta-endorphin enhances B cell proliferation. However, at suboptimal mitogen concentrations, beta-endorphin suppressed the proliferative responses to all the mitogens, suggesting that it is capable of modulating both T and B cell activity. Alpha-endorphin suppresses antibody formation in human lymphocytes at both the T and B cell level, while beta-endorphin enhances anti-herpes antibodies in a biphasic manner [Heijnen, *et al.*, 1986; Williamson, *et al.*, 1988]. Beta-endorphin has also been found to enhance natural killer cell activity in a biphasic manner which is a mirror image of the effects on antibody production, therefore suggesting that the immunomodulatory effects of beta-endorphin may be mediated by effects on natural killer suppressor-inducer cells [Williamson, *et al.*,

1988]. Therefore, beta-endorphin may modulate equine lymphocyte proliferative response by activating natural killer suppressor-inducer cells in a biphasic manner. The subset of natural killer cells which are activated may depend on the concentration of beta-endorphin or the receptor population of the respective lymphocyte subsets.

All experiments were performed using lymphocytes from conditioned or trained horses. Previous studies demonstrated decreased affinity of the receptor for beta-endorphin in fit horses [Chapter 6]. The decreased affinity of the receptor in fit horses may have decreased the ability of beta-endorphin to modulate proliferation in maximally stimulated cells. Therefore, the actual response of the cells to beta-endorphin may have been masked. These experiments also revealed a great deal of individual variation which has also been noted by other investigators. Heijnen, *et al.* [1987] commented that the modulatory activity of beta-endorphin was dependent upon, not only concentration, but on the donor of the cells, and that repeated testing of individuals revealed identical response patterns. It has also been noted that modulation of natural killer cell activity may be dependent upon certain "low responder" and "high responder" populations [Oleson and Johnson, 1988]. Therefore, the variation noted in these assays may be due to inherent capacities of each donor, as well as the activity state of the individual cells. The modulatory ability of beta-endorphin may be dependent on the expression of the opioid receptor on the lymphocyte, which may be related to the activity state of the cell. As activation occurs, i.e. stimulation by a mitogen, opioid receptor expression may increase and therefore increase the ability of beta-endorphin to modulate lymphocyte function. However, there may be a loss of sensitivity or masking effect in maximally stimulated

cells.

The effect of naloxone on the proliferative response of equine lymphocytes to various mitogens yielded interesting results. Naloxone is thought to be a pure opiate antagonist with no activity of its own at opioid receptors [Martin, 1984]. However, naloxone also has apparent agonist actions similar to those seen with morphine in a variety of *in vivo* and *in vitro* studies [Sawynok, *et al.*, 1979]. In the present experiments, naloxone both suppressed and enhanced lymphocyte proliferation responses significantly. At optimal ConA concentrations, naloxone plus beta-endorphin enhanced the response, although it did not differ significantly from the response to beta-endorphin alone. At suboptimal doses of all the mitogens, naloxone suppressed the proliferative response significantly. There was no difference between the response of naloxone and beta-endorphin and the addition of beta-endorphin and naloxone together did not enhance the response of either. Therefore, it is conceivable that beta-endorphin and naloxone were acting at the same receptor since the response did not change, or become additive, when the two were added together. Earlier studies which suggested that naloxone possessed agonist actions generally demonstrated agonist activity at high doses and antagonist activity at low doses [Sawynok, *et al.*, 1979]. The results reported in this study are consistent with this report as the naloxone dose used in this study was very high (10^{-5}M). Therefore, it would be interesting to see if lower naloxone doses would exhibit antagonist actions in this study. However, the effects of naloxone on equine lymphocyte proliferation could also result from a nonspecific physiologic blockade of nonopioid receptors responsible for mitogen activity. These results, when analyzed in conjunction

with the results of the binding studies [Chapters 5 and 6], suggest that beta-endorphin is acting at an opioid receptor when modulating lymphocyte activity and that naloxone may also possess similar activity at this receptor.

In conclusion, beta-endorphin modulates equine lymphocyte mitogen induced proliferative responses through interaction with an opioid receptor. The conflicting results obtained at optimal and suboptimal doses of the mitogens may be due to individual responses to beta-endorphin, perhaps a result of the activity state or opioid receptor expression of the lymphocyte population of the individual, or may be indicative of a biphasic response to beta-endorphin. Naloxone also modulated the proliferative responses of equine lymphocytes, which suggests that naloxone possesses agonist activity at the opioid receptor. However, nonspecific effects of naloxone when used at high doses can not be eliminated. These results suggest a biological role for circulating beta-endorphin in the horse and support the theory of an immune system -- central nervous system regulatory circuit.

CHAPTER EIGHT

SUMMARY AND CONCLUSIONS

Beta-endorphin is released into the peripheral circulation in response to maximal exercise. Questions as to the significance of peripherally released beta-endorphin during exercise have led to research into its role in mood enhancement, analgesia, food intake suppression and reproductive dysfunction [Sforzo, 1989]. However, identification of opioid receptors on human and murine leukocytes, along with modulation of immune cell function by endogenous opioids, suggests that endogenous opioids may be potential mediators of stress-induced immunomodulation [Sibinga and Goldstein, 1988]. Although elevated beta-endorphin levels following exercise have been demonstrated in the horse [Li and Chen, 1987], the role of peripherally circulating beta-endorphin in the horse has not been examined. In addition, the effects of exercise and physical conditioning on immune function have not been examined, nor has the role of beta-endorphin in conditioning effects been assessed.

Beta-endorphin was found to vary diurnally in the horse in a manner similar to that seen in humans, with peak levels at 0900 hours. Increased pain threshold, increased heart rate and increased pupil diameter were also observed at the time beta-endorphin concentrations were highest. These findings correlate well with the effects of morphine in the horse [Muir, *et al.*, 1980], and suggest that beta-endorphin may modulate a number of physiologic responses. Based on earlier studies which suggested that endogenous opioid systems exhibit tonic activity, it was concluded that optimal experimental times

would be when basal beta-endorphin levels were the highest, i.e. 0900 hours. Therefore, all further experiments were conducted between 0800 and 1000 hours.

During a 24 hour period, heart rate was the highest at 0900 hours, as was beta-endorphin, suggesting that beta-endorphin affects cardiovascular function. This is supported by earlier studies which suggest opioid peptides affect cardiovascular function [Sforzo, 1989]. However, administration of naloxone in the first exercise study did not alter the cardiovascular response to exercise, suggesting that beta-endorphin, or other endogenous opioids, does not mediate cardiovascular responses to exercise. Naloxone administration also does not alter the cardiovascular response to exercise in humans, which supports the theory that endogenous opioids are not involved in the cardiovascular response to exercise. Nevertheless, the use of naloxone as the sole method of determining opioid receptor involvement makes drawing appropriate inferences difficult. Naloxone exhibits a number of agonist activities as well as antagonist activity [Sawynok, *et al.*, 1979] and one investigator was able to demonstrate a significant, albeit minor, decrease in maximal heart rate in women with opiate blockade [Cummings and Wheeler, 1987]. Therefore, a role of beta-endorphin and other endogenous opioids in the cardiovascular response to exercise cannot be eliminated. In addition, although beta-endorphin may not be involved in acute cardiovascular responses to exercise, this does not rule out a role for endogenous opioids in cardiovascular adaptations to chronic exercise, i.e. physical conditioning, or in diurnal variation of heart rate.

Beta-endorphin release in response to maximal exercise in the horse appeared to be correlated with both exercise intensity and physical conditioning. Analgesia

measurements, combined with evaluation of heart rate and lactate measurements, were used to assess the degree of exertional stress. Analgesia was seen following exercise in the unfit horse, along with significantly higher heart rates. Although analgesia was noted after an increase in exercise duration in the fit horse, it was not as great as that seen in the unfit horse following a less strenuous exercise test. These results suggest that the unfit horse experienced a greater degree of exertional stress than did the fit horse, even when the fit horse was subjected to an exercise test of higher exercise intensity. The greater amount of exertional stress, however, did not result in higher beta-endorphin concentrations, as beta-endorphin concentrations were higher in the fit horse after a 1000 m exercise test than in the unfit horse following a 400 m exercise test.. Therefore, it was concluded that beta-endorphin release was associated to a greater degree with exercise intensity rather than exertional stress. Nonetheless, beta-endorphin release in response to exercise can be enhanced by other physical stressors, such as hot ambient temperature and dehydration [Cummings and Wheeler, 1987], implying that many other factors contribute to the response of the opioid system to exercise.

A strong positive correlation between beta-endorphin concentrations and lactate concentrations following exercise support the theory that beta-endorphin release is linked with onset of blood lactate accumulation. Several studies in humans have also noted a correlation between beta-endorphin and lactate concentrations [Sforzo, 1989] and speculation about a mechanistic link between beta-endorphin release and the onset of blood lactate accumulation can be found in the literature [DeMeirler, *et al.*, 1986]. Although exercise intensity and the onset of blood lactate accumulation have been linked

with the release of beta-endorphin, a number of factors may be involved. Other possible stimulatory stressors which have been suggested are hypoglycemia and stimulation of neural pathways [DeMeirler, *et al.*, 1986].

Beta-endorphin concentrations were positively correlated with pain threshold measurements in the diurnal study. Analgesia was also noted following exercise, but did not correlate with beta-endorphin concentrations, as beta-endorphin concentrations were elevated when there was no analgesia. Also, beta-endorphin levels were higher in fit horses than in unfit horses although the unfit horses had a greater degree of analgesia. However, naloxone reversed the analgesia noted following exercise. These results suggest that endogenous opioids are involved with analgesia, but do not support the opinion that peripherally circulating beta-endorphin modulates pain perception. The fact that beta-endorphin concentrations correlated well with analgesia measurements in the diurnal study may be explained by diurnal fluctuation of endogenous opioids, which originate in the central nervous system. The lack of correlation between pain threshold measurements and beta-endorphin concentrations following exercise does not, however, eliminate a role for beta-endorphin in pain modulation. Pain modulation following exercise may result from interactions between a number of factors, such as beta-endorphin release, muscle afferent discharges, degree of exertional stress and perhaps, psychological factors. Therefore, beta-endorphin could be one factor which contributes to the modulation of pain perception.

The effects of physical conditioning on the opioid response to exercise has generated conflicting reports [Sforzo, 1989]. A nine week training period resulted in an

attenuated beta-endorphin output following exercise in the horse, although the difference was noted at one time (thirty minutes following exercise). These results suggest that beta-endorphin concentrations return to baseline faster in the fit horse. However, increased exercise intensity resulted in significantly higher levels of beta-endorphin following exercise, suggesting that fit horses may accumulate beta-endorphin in a manner similar to the ability of trained individuals to accumulate great lactate concentrations [Sforzo, 1989]. Nonetheless, the greater beta-endorphin and lactate concentration accumulation noted in trained individuals may be a function of a degree of exercise intensity which is attainable only by trained athletes.

The effects of naloxone administration on the response to exercise in the horse generated some interesting results. While naloxone reversed the analgesia noted in the fit horse, it not only reversed but caused hyperalgesia in the unfit horse. Also, naloxone administration resulted in prolonged increases in beta-endorphin levels in both fit and unfit horses. The prolonged release of beta-endorphin following naloxone administration implies that opioid receptors are involved in modulating the response to exercise, perhaps through autoreceptors at the hypothalamic or pituitary level. The fact that the analgesic response to naloxone administration in the unfit horse differed from that in the fit horse indicates a change in opioid receptor function following athletic conditioning. The lack of hyperalgesia in the fit horse implies a down regulation of the receptor due to the effects of chronic exercise. A decrease in receptor number or affinity would explain the lack of sensitivity to the effects of naloxone.

A growing number of reports support the view that physical exercise can modulate

immune function. Acute exercise causes a marked increase in the number of circulating lymphocytes, suppresses lymphocyte proliferative responses, reduces the ratio of T helper cells to T suppressor cell, and reduces antibody production [Keast, *et al.*, 1988]. These reports imply that a temporary immune suppression occurs following acute exercise. However, the effects of chronic exercise on immune function have not been evaluated. These reports, coupled with reports of opioid receptors on leukocytes, suggest that beta-endorphin may be a mediator in the modulation of immune function following exercise.

Highly specific, saturable receptors for beta-endorphin were identified on equine lymphocytes. Binding to the receptors took place in a time, temperature and pH dependent manner. Scatchard/Rosenthal analysis of the saturation isotherm revealed a binding site with a K_d of 17 pM and B_{max} of 0.538 fmoles/10^6 cells. Competition-inhibition curves revealed an opioid receptor, with mu and/or delta receptor specificity. The slopes of the competition curves revealed a complex ligand-receptor interaction which, coupled with the ability of both mu and delta agonists to displace radiolabeled beta-endorphin, suggests that the receptor may be a mu/delta opioid receptor complex with interacting mu and delta binding sites. However, the Scatchard analysis of the saturation binding data demonstrated a simple interaction between one ligand and a single population of receptors. On the other hand, the number of radioligand concentrations used in the saturation may not have been sufficient for detection of a more complex interaction. Therefore, this discrepancy may be resolved by increasing the number of radioligand concentrations used in the saturation isotherm.

While the identification of opioid receptors on equine lymphocytes suggests a role

for beta-endorphin in the immune response to exercise, it is not conclusive. Therefore, binding parameters of beta-endorphin receptors on equine lymphocytes were analyzed following both acute and chronic exercise. Beta-endorphin receptors showed enhanced affinity for the ligand following acute near maximal exercise, coupled with decreased receptor number. The decrease in receptor number following exercise may indicate that receptor internalization was occurring in response to ligand-receptor interactions. These changes in receptor binding parameters provide further evidence that beta-endorphin may help to modulate the immune response to exercise. Beta-endorphin receptor binding also showed a decreased affinity for the ligand following a 20 week conditioning period, as compared to a population of sedentary horses. However, the total number of receptors did not change with chronic exercise. The number of receptors decreased following acute exercise, but did not change in resting populations of fit and unfit horses. This supports the theory that the decrease in receptor number following acute exercise is due to receptor internalization, and is a transient response to increased beta-endorphin levels and therefore would not be seen in resting horses.

The decreased affinity of the receptor following chronic exercise suggests that there is a down regulation of the receptor in response to continual high levels of circulating beta-endorphin. These results support the conclusions drawn from the effects of naloxone administration on pain threshold. It was concluded that the hyperalgesia noted in the unfit horse, which was not seen in the fit horse following naloxone administration, was due to a down regulation of opioid receptors following chronic exercise. Since peripherally circulating beta-endorphin cannot be definitively linked with

pain perception, the role of central opioid receptors cannot be eliminated. Therefore, these results imply that a down regulation of opioid receptors occurs following chronic exercise, not only on leukocytes, but perhaps in the central nervous system. Therefore, the effects of chronic exercise may be mediated, in part, by endogenous opioids, both at the central and peripheral levels.

Acute exhaustive exercise appears to suppress immune function in humans [Keast, *et al.*, 1988]. Beta-endorphin also has been reported to suppress lymphocyte proliferative responses in humans [Sibinga and Goldstein, 1988]. If beta-endorphin suppresses immune function in the horse, then beta-endorphin may modulate the suppression of immune function following acute exercise. The changes in receptor binding following acute and chronic exercise in the horse suggest that beta-endorphin may be involved in suppressing immune function following acute exercise in the horse while physical conditioning may attenuate this response.

To confirm the ability of beta-endorphin to modulate equine immune function, the effects of beta-endorphin on equine lymphocyte proliferative responses were evaluated. Beta-endorphin significantly suppressed the proliferative responses of equine lymphocytes to the mitogens ConA, PHA and PWM at suboptimal concentrations. Although beta-endorphin appeared to enhance proliferation at optimal mitogen concentrations, this was most likely an artifact resulting from large population variance. Naloxone is generally thought to be a pure antagonist, acting primarily at the mu opioid receptor [Martin, 1984]. However, naloxone also appears to have weak agonist activity at opioid receptors when used in high doses [Sawynok, *et al.*, 1979]. In this study, naloxone suppressed the

proliferative response to all three mitogens, and the addition of beta-endorphin and naloxone was not different from either beta-endorphin or naloxone added alone. Therefore, the effects of beta-endorphin and naloxone were not additive, which suggests that they were acting through the same receptor. These results also suggest that naloxone may produce agonist effects when used in high concentrations.

The ability of beta-endorphin to suppress equine lymphocyte proliferative responses to mitogens supports the view that beta-endorphin mediates exercise-induced alteration of immune function. Therefore, since beta-endorphin suppresses equine lymphocyte proliferative responses, it is reasonable to conclude that beta-endorphin released during exercise may be, in part, responsible for the temporary suppression of immune function seen following acute exercise. Beta-endorphin influences immune function in the horse through interaction with specific receptors on lymphocytes. The change in binding parameters supports the popular idea that a fit individual is a healthier individual. The downregulation or decreased sensitivity of the receptor in the fit horse implies that physical conditioning improves the immune response to stress, such as exertional stress. Nonetheless, beta-endorphin can not be considered as the sole mediator of the effects of exertional stress on immune function. Adrenocorticotropin is co-released from the pituitary with beta-endorphin and stimulates the production of cortisol [Guillemin, *et al.*, 1977]. Cortisol levels are increased following exercise and cortisol has also been shown to suppress mitogen-induced proliferative responses [Keast, *et al.*, 1988]. Catecholamine levels also increase during exercise and have been shown to modify the number and function of circulating lymphocytes [Keast, *et al.*, 1988]. Therefore, while

the results from this study confirm a role for beta-endorphin in exercise-induced immunosuppression, it cannot be considered the only possible mediator.

The ability of beta-endorphin to modulate equine lymphocyte proliferative response through interaction with a highly specific, saturable receptor implicates a role for beta-endorphin in equine immune responses to exercise. The changes which occur as a result of chronic exercise suggest that beta-endorphin is also involved in adaptations to chronic exercise and that these adaptations result in improved immune function. However, until the effects of acute and chronic exercise on equine immune function are evaluated, a role for beta-endorphin in equine immune responses to exercise cannot be confirmed. Beta-endorphin modulation of immune function through opioid receptors on equine lymphocytes also supports the hypothesis of a bidirectional communication system between the immune system and the central nervous system. Here again, evidence for production of endogenous opioids by cells of the immune system would be required to confirm this theory. In conclusion, these data support the hypothesis that beta-endorphin is a mediator of acute exercise-induced immune responses and is also involved in adaptations of the immune response to chronic exercise.

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DOCTORAL EXAMINATION AND DISSERTATION REPORT

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Major Field: Veterinary Medical Sciences (Pharmacology Option)

Title of Dissertation: Beta-endorphin Receptors on Equine Lymphocytes: Relationship to Immune Function and Exercise

Approved:

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